MODIFICATION OF POLYSACCHARIDE CONTAINING MATERIALS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to methods and compositions for altering the structural, chemical, physical and mechanical properties of polysaccharide materials using biological crosslinking agents based on multimeric structures of polysaccharide binding domains fused or linked to a biological or chemical entity and the resulting biological compositions. The invention is exemplified by the use of a cellulose binding domain (CBD) fusion protein containing two cellulose binding domains, a cellulose binding domain-Protein A-Ab complex or a S-peptide-cellulose binding domain-S-protein fusion to enhance mechanical properties, such as wet strength, of tissue paper, filter paper and cotton yarn.

Polysaccharides are ubiquitous, stable structural components found in nature. Many organisms use polysaccharides as structural material inside and outside of their cells to provide 3-dimensional shape and surface structure. The structural integrity of polysaccharides from natural sources is often retained after the isolation of the polysaccharide, allowing it to be used for a variety of commercial purposes. Owing to their desirable physical characteristics polysaccharides have also been produced by synthetic methods for commercial purposes. In either case, polysaccharides such as celluloses from either synthetic or non-synthetic sources comprise the raw material for a variety of commercially important products such as paper pulp, and textile fibers.

The paper manufacturing process conventionally includes four main steps: forming an aqueous suspension of cellulosic fibers, commonly known as pulp; adding various processing and paper enhancing materials, such as strengthening and/or sizing materials to the pulp slurry; sheeting the paper by pouring the resulting suspension over forming fabric which filters out most of the water and drying the fibers to form a desired cellulosic web; and post-treating the web after an initial drying of the paper to provide various desired characteristics to the resulting paper, including surface application of sizing materials to increase the dry strength of the paper. Those additives applied to the pulp in an aqueous slurry are known as wet-end additives and include retention aids to retain fines and fillers, for example, alum, polyethylene imine, cationic starches and the like; drainage aids, such as polyethylene imine; defoamers; and pitch or additives such as microfibers and adsorbent fillers. Other wet-end additives include polymers such as, cationic polyarylamides and poly(amide amine/epichlorohydrin) which are added to improve wet strength as well as dry strength of the paper. Starch, guar gums, and polyacrylamides are also added to yield dry strength improvements. Sizing agents are occasionally added to impart hydrophobic character to the hydrophilic cellulosic fibers. These agents are used in the

manufacture of paper for liquid containers, for example, milk or juice, paper cups and surfaces printed by aqueous inks where it is desired to prevent the ink from spreading. Such sizing agents include rosin sizes derived from pine trees, wax emulsions and, more recently, cellulose-reactive sizes. The application of additives to paper after an initial drying of the sheet by spraying, capillary sorption, immersion, roll-coating and the like, is often referred to as a dry-end addition. Poly(vinyl alcohol), acrylic or vinyl acetate emulsions, starches, sizing agents, polyurethanes, and SBR latex are commonly added at the dry end.

A major product of the paper industry is corrugating medium, the middle fluting paper used in corrugated containers. Starch makes up 2-5% of the total weight of fluting paper. Various techniques have been used to improve the wet strength of corrugating medium, including the use of chemical crosslinkers, such as formaldehyde resins, or the application of hydrophobic materials, such as wax. However, the addition to or treatment of paper with such compounds has been largely discontinued due to the negative impact of these compounds on recyclability of the treated paper. Other techniques used have employed more expensive raw materials such as semichemical pulp in order to increase the weight and strength of the paper per square meter. This latter approach leads to increased cost of both starting materials and the manufacturing process itself.

The processing of cellulosic material, as for example cotton fiber into a textile fabric, like paper making, also involves several steps: spinning of the fiber into a yarn; construction of woven or knit fabric from the yarn and subsequent preparation, dyeing and finishing operations. Woven goods are the prevalent forms of textile fabric construction. The yarns generally are sized in a size box, then the water is removed on steam cans and the yarns formed into a sheet which is run across bust rods to break the sheet back into individual yarns. The yarns are then woven, which is done by weaving a filling yarn between a series of warp yarns. The sub-steps involved in preparation are desizing, scouring and bleaching. A one-step combined scour/bleach process is also used in the industry.

Various compounds are used as sizing agents for warp yarns to prevent breakage of the yarn during weaving. A good yarn sizing agent is one which forms a film with sufficient strength to provide protection to the yarn being sized but is not so strong that the yarn will break under the size film. The sizing agents are placed on the warp yarns prior to weaving to provide strength and to protect the yarns from abrasion. Traditional sizing agents for cotton-containing yarns have generally included film formers such as starch, derivatives of starch, polyvinyl alcohol, polyester resins, waxes, acrylic polymers and copolymers and their salts, wetting agents, antistatic agents, and combinations thereof. The conventional thermosetting resin systems,

either postcured or precured, result in embrittlement and reduction of mobility of the microstructural units of cellulosic fibers to such an extent that abrasion resistance, breaking strength, and tearing strength often are seriously impaired. Abrasion resistance is often reduced by 75-85%, breaking strength by 50-60%, and tearing strength by about 50%. Furthermore, if the cellulosic fiber-containing yarn is sized by the conventional methods described above, it is difficult to completely desize the sized yarn. Even if the sized yarn is completely desized, the desizing process is complicated or expensive.

Recently, it has become an increasingly important requirement that desizing be effected in a simple process, without pollution. It therefore is of interest to develop additives for polysaccharide containing materials such as paper and textiles which decrease or avoid the use of potentially toxic chemical crosslinkers, and which are cost and time effective to use.

Relevant Literature:

Disruption of cellulose fibers by the binding domain of a bacterial cellulase is described by Din *et al.* (1991) *Bio/Technology* 9: 1096-1099. Kim *et al.* (1993, *Protein Science* 2: 348-356) describe a recombinant fusion protein having a S-peptide carrier, an oligopeptide spacer having a protease recognition sequence, and a galactosidase target. Expression of a fusion protein of heparinase I (*ex Flavobacterium heparinum*) fused to either the N- or C- terminal of the CBD of *C. cellulovorans* was described by Shpigel *et al.* (1999) *Biotech. Bioeng.* 65:17-23.

U.S. PAT. NO. 5,137,819 to Kilburn *et al.* discloses the preparation of fusion proteins which include a cellulase substrate binding region and their use in immobilization and purification of polypeptides. U.S. PAT. NO. 5,928,917 to Kilburn *et al.* discloses conjugates of a non-protein chemical moiety and a polypeptide having a cellulose binding region. Polysaccharide binding proteins and conjugates are described in U.S. PAT. NO. 5,962,289 to Kilburn *et al.* U.S. PAT. NO. 5,821,358 to Gilkes *et al.* discloses methods and compositions for the modification of polysaccharide structures, for example, cotton and ramie fibers, using binding domains and/or catalytic domains from polysaccharidases.

U.S. PAT. NO. 5,837,814 to Shoseyov *et al.* discloses a CBD having a high affinity for crystalline cellulose and chitin, together with fusion products of the CBD and a second protein. Applications for the CBD and the fusion products, including: drug delivery, affinity separations, and diagnostic techniques are also disclosed. See also, U.S. PAT. NO. 5,719,044; U.S. PAT. NO. 5,496,934; and U.S.5,856,201; all to Shoseyov *et al.*, the contents of each of which are incorporated by reference herein.

A review of the utility of paper additives is given by B.B. Spence *Encyclopedia of Polymer Science and Technology*, Second Edition, Wiley-Interscience, Vol. 10, pgs. 761-786, New York (1987).

SUMMARY OF THE INVENTION

The present invention is directed to compositions and methods for cross-linking and/or functionalizing polymeric or polysaccharide materials using compositions comprising at least one polysaccharide binding domain. Compositions of the invention include polysaccharide binding domain (PBD) fusion proteins, PBD coupler units, PBD functional moieties and polysaccharides modified using these compositions. A PBD coupler unit of the invention includes one, two or more PBDs, each of which is capable of independently binding to a polysaccharide, and optionally one or more linker unit coupled between the PBDs. The method includes the step of contacting a polysaccharide structure with a sufficient amount of a PBD fusion protein under conditions and for a time sufficient to modify one or more characteristic of a polysaccharide material comprising a polysaccharide structure. The methods and compositions find use in producing polysaccharide containing materials with altered mechanical, chemical, electrical and/or physical properties.

According to one aspect of the present invention there is provided a process of manufacturing a polysaccharide containing material having at least one desired structural, chemical, physical, electrical and/or mechanical property, the method comprising the step of contacting polysaccharide structures of the polysaccharide containing material with a polysaccharide binding domain containing composition before, during and/or after processing the polysaccharide structures into the polysaccharide containing material, thereby manufacturing the polysaccharide containing material having the desired structural, chemical, physical, electrical and/or mechanical property.

According to another aspect of the present invention there is provided a composition-of-matter comprising a polysaccharide containing material including polysaccharide structures; and a polysaccharide binding domain containing composition being bound to the polysaccharide structures of the polysaccharide containing material, providing the polysaccharide containing material with at least one desired structural, chemical, physical, electrical and/or mechanical property.

According to further features in preferred embodiments of the invention described below, contacting the polysaccharide structures of the polysaccharide containing material with the polysaccharide binding domain containing composition is effected before processing the polysaccharide structures into the polysaccharide containing material.

According to still further features in the described preferred embodiments contacting the polysaccharide structures of the polysaccharide containing material with the polysaccharide binding domain containing composition is effected during processing the polysaccharide structures into the polysaccharide containing material.

According to still further features in the described preferred embodiments contacting the polysaccharide structures of the polysaccharide containing material with the polysaccharide binding domain containing composition is effected after processing the polysaccharide structures into the polysaccharide containing material.

According to still further features in the described preferred embodiments the polysaccharide containing material is selected from the group consisting of a paper, a textile, a yarn and a fiber.

According to still further features in the described preferred embodiments the structural property is selected from the group consisting of a predetermined level of cross-links between polysaccharide structures of the polysaccharide containing material, a predtermined aggregation of the polysaccharide structures of the polysaccharide containing material and a predetermined surface texture of the polysaccharide containing material.

According to still further features in the described preferred embodiments the chemical property is selected from the group consisting of a predetermined hydrophobicity, a predetermined hydrophylicity, a predetermined wet-ability, a predetermined chemical reactivity, a predetermined photochemical reactivity, a predetermined functionality and a predetermined surface tension.

According to still further features in the described preferred embodiments the physical property is selected from the group consisting of a predetermined Young's modulus, a predetermined strain at maximum load, a predetermined energy to break point, a predetermined water absorbency, a predetermined swellability and a predetermined toughness.

According to still further features in the described preferred embodiments the electrical property is selected from the group consisting of a predetermined surface charge and a predetermined electrical conductivity.

According to still further features in the described preferred embodiments the mechanical property is selected from the group consisting of a predetermined tensile strength, a predetermined resistance to shear, a predetermined abrasion resistance, a predetermined frictional coefficient, a predetermined elasticity and a predetermined wet strength.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide

binding domain and at least one additional polysaccharide binding domain covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and another protein covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a hydrophobic group covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a hydrophilic group covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a biological moiety covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and an enzyme covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and an chemical reactive group covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and an chemical photoreactive group covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a lipase covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a lacase covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a protein A-antibody covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a peptide covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a polypeptide covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a hydrocarbon or a hydrocarbon derivative covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a fatty acid derivative covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and an electrically charged moiety covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and an ionic moiety covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a silicon binding moiety covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a polymer binding moiety covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a metal covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a metallothionein-like protein covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and ferritin covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a metal binding moiety covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a bacterial siderophores covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a metallothionein covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a thiol group covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and an aldehyde covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a maleimide covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a hydrazide covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and an epoxide covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a carbodiimide covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain and a phenylazide covalently coupled thereto.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain which is a cellulose binding domain.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain which is a starch binding domain.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain capable of binding to cellulose.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain capable of binding to starch.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain capable of binding to chitin.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide

binding domain which is a glucan-binding domain, e.g., a β-1,3-glucan-binding domain.

According to still further features in the described preferred embodiments the polysaccharide binding domain containing composition includes a polysaccharide binding domain which includes *streptococcal* glucan-binding repeats.

According to yet another aspect of the present invention there is provided a composition-of-matter comprising a polysaccharide containing material including polysaccharide structures; and a polysaccharide binding domain containing composition being bound to the polysaccharide structures of the polysaccharide containing material, the polysaccharide binding domain containing composition including at least two covalently coupled polysaccharide binding domains forming a polysaccharide binding domain coupler cross linking the polysaccharide structures of the polysaccharide containing material.

According to still another aspect of the present invention there is provided a composition-of-matter comprising a polysaccharide containing material including polysaccharide structures; and a polysaccharide binding domain containing composition being bound to the polysaccharide structures of the polysaccharide containing material, the polysaccharide binding domain containing composition including at least one polysaccharide binding domain and a functionalizing moiety being covalently coupled thereto, the at least one polysaccharide binding domain attaching the functionalizing moiety to the polysaccharide structures of the polysaccharide containing material.

According to an additional aspect of the present invention there is provided a composition-of-matter comprising a polysaccharide containing material including polysaccharide structures; and a polysaccharide binding domain containing composition being bound to the polysaccharide structures of the polysaccharide containing material, the polysaccharide binding domain containing composition including at least one polysaccharide binding domain and a hydrophobic moiety being covalently coupled thereto, the at least one polysaccharide binding domain attaching the hydrophobic moiety to the polysaccharide structures of the polysaccharide containing material.

According to yet an additional aspect of the present invention there is provided a composition-of-matter comprising a polysaccharide containing material including polysaccharide structures; and a polysaccharide binding domain containing composition being bound to the polysaccharide structures of the polysaccharide containing material, the polysaccharide binding domain containing composition including at least one polysaccharide binding domain and a hydrophilic moiety being covalently coupled thereto, the at least one polysaccharide binding domain attaching

the hydrophilic moiety to the polysaccharide structures of the polysaccharide containing material.

According to still an additional aspect of the present invention there is provided a composition-of-matter comprising a polysaccharide containing material including polysaccharide structures; and a polysaccharide binding domain containing composition being bound to the polysaccharide structures of the polysaccharide containing material, the polysaccharide binding domain containing composition including at least one polysaccharide binding domain and a chemical reactive moiety being covalently coupled thereto, the at least one polysaccharide binding domain attaching the chemical reactive moiety to the polysaccharide structures of the polysaccharide containing material.

According to a further aspect of the present invention there is provided a composition-of-matter comprising a polysaccharide containing material including polysaccharide structures; and a polysaccharide binding domain containing composition being bound to the polysaccharide structures of the polysaccharide containing material, the polysaccharide binding domain containing composition including at least one polysaccharide binding domain and a photo-chemical reactive moiety being covalently coupled thereto, the at least one polysaccharide binding domain attaching the photo-chemical reactive moiety to the polysaccharide structures of the polysaccharide containing material.

According to yet a further aspect of the present invention there is provided a composition-of-matter comprising a polysaccharide binding domain coupler including at least two covalently coupled polysaccharide binding domains.

According to still a further aspect of the present invention there is provided a nucleic acid construct comprising a polynucleotide encoding a fusion protein including at least two polysaccharide binding domains. Preferably, the nucleic acid construct further comprising at least one additional polynucleotide encoding at least one linker peptide coupling the at least two polysaccharide binding domains.

According to a further aspect of the present invention there is provided a process of manufacturing a polysaccharide containing material having at least one desired structural, chemical, physical, electrical and/or mechanical property, the method comprising the step of contacting polysaccharide structures of the polysaccharide containing material with a polysaccharide binding domain, during and/or after processing the polysaccharide structures into the polysaccharide containing material, and thereafter covalently coupling at least one moiety or group to the polysaccharide binding domain, thereby manufacturing the polysaccharide containing material having the desired structural, chemical, physical, electrical and/or mechanical property.

The present invention successfully addresses the shortcomings of the presently known configurations by providing processes and reagents for manufacturing superior polysaccharide structures containing materials such as papers and textiles.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

Fig. 1A is a schematic representation of pET-CBD plasmid.

Figs.1B-C show the nucleotide sequence (SEQ ID NO:1) and the amino acid sequence (SEQ ID NO:2) of CBDclos.

Fig. 1D is a schematic representation of pET-CBD-180.

Figs. 1E-G show the nucleotide sequence (SEQ ID NO:3) and the amino acid sequence (SEQ ID NO:4) of CBD-180 along with restriction endonuclease recognition sites.

Fig. 2A is a schematic representation of pET-CCP-180 containing one copy of CBD-180 and one of CBD fused in frame thereto.

Figs. 2B-E show the nucleotide sequence (SEQ ID NO:5) and the amino acid sequence (SEQ ID NO:6) of CCP (cellulose cross linking protein) along with restriction endonuclease recognition sites.

Fig. 3A is a schematic representation of pET-ProtA-CBD.

Figs. 3B-G show the nucleotide sequence (SEQ ID NO:7) and the amino acid sequence (SEQ ID NO:8) of ProtA-CBD.

Fig. 4A is a schematic representation of pET29-Spep-CBD-Sprot.

Figs. 4B-G show the nucleotide sequence (SEQ ID NO:9) and the amino acid sequence (SEQ ID NO:10) of Spep-CBD-Sprot.

Fig. 5A schematically represents a cellulose cross-linking protein having two domains for cellulose binding per molecule.

Fig. 5B schematically represents the cellulose cross-linking protein of Fig. 5A, wherein one cellulose binding domain is bound to a first polymeric structural unit, and a second cellulose binding domain is bound to a second polymeric structural unit.

Fig. 6 schematically represents a generic CBD coupler unit.

Figs. 7A-C each schematically represent various embodiments of a CBD coupler unit. Fig. 7A shows a CBD coupler unit having a pair of terminal CBDs linked by a linker unit which includes a pair of starch binding domains each coupled to a CBD, and a starch moiety coupled to both starch binding domains. Fig. 7B shows a CBD coupler unit having a pair of terminal CBDs linked by a linker unit which includes a plurality of CBDs, each of which is coupled to an adjacent CBD via a JUN/FOS bridge. Fig. 7C shows a CBD coupler unit having a pair of terminal CBDs linked by a large protein moiety.

Fig. 8 schematically represents a CBD functional moiety (CBDC) having at least one CBD and a functional moiety (FM) attached thereto.

Figs. 9A-C schematically represent various ways in which a CBD coupler unit can interact with, and bind to, a polymeric structural unit.

Figs. 10A-D are bar graphs of Young's modulus, strain at maximum load, energy to break point and toughness, respectively, for control, CBD-treated, and CCP-treated paper strips.

Fig. 11 schematically represents a yarn coating apparatus used for treating the yarn with test formulations.

Figs. 12A-B are bar graphs of Young's modulus and strain at maximum load, respectively, for control, CCP-treated, ProtA-CBD treated, and Ab-ProtA-CBD treated yarn.

Fig. 13 shows a photograph of the results of expression of S-protein-CBD-S-peptide (SCS) in *E.coli*. Protein marker (lane 1), total *E.coli* proteins before induction with IPTG (lane 2) total *E.coli* proteins after induction with IPTG (lane 3) and inclusion bodies containing the SCS protein (lane 4).

Fig. 14 shows a Young's modulus map of the results of treating Whatman papers with CBD, CCP, or SCS. All measurements were taken at 23 °C, 65 % relative humidity.

Fig. 15 shows the energy to break points of CBD, CCP, and SCS treated Whatman papers. All measurements were taken at 23 °C, 65 % relative humidity.

Fig. 16 shows the results of a toughness test of CBD, CCP, and SCS treated Whatman papers. All measurements were taken at 23 °C, 65 % relative humidity.

Fig. 17 shows the stress at maximum load of CBD, CCP, and SCS treated Whatman papers. All measurements were taken at 23 °C, 65 % relative humidity.

Fig. 18 shows typical stress versus strain curves of preformed Whatman papers treated with CBD or CCP. All measurements were taken at 23°C, 65% relative humidity and at a constant deformation rate of 20 mm/min. Squers – Control; Circles – 2.5 mg/ml CBD; triangles – 2.5 mg/ml CCP.

Fig. 19 shows water-absorption time of preformed Whatman papers treated with CBD or CCP at different concentrations. All measurements were taken at 23°C . Distilled water (10 µl) was pipetted onto treated papers and the time to full absorption was measured in seconds (control – solid bar; CBD – stippled bars; CCP – open bars).

Fig. 20 shows time-lapse photograps of water absorption on preformed Whatman pape treated with CCP. Water droplets (20 µl each) were dripped onto CCP-treated paper, and pictures were taken every 25 ms. The first frame (A) was taken before the water made contact with the paper. Frames B to E ware taken after 2, 4, 6, and 8 minutes, respectively. The last frame (F) was taken on non-treated paper 25 ms after water came in contact with the paper. Water absorption was visualized using an optical contact angle meter.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the subject invention, methods and compositions are provided for altering surface, chemical, electrical, and mechanical properties of polysaccharide materials.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Polysaccharide structures such as fibers and filaments are treated with PBD-containing compositions during or after processing of the structures into polysaccharide containing materials, thereby cross-linking and/or functionalizing the polysaccharide structures and/or their products such as textiles and paper. The PBD-containing compositions function as versatile biological crosslinkers, the structure of which can be varied to accommodate a desired result such as increased elasticity or hydrophobicity of a polysaccharide containing end product. The biological crosslinkers are multimeric proteins containing at least one PBD, such as a cellulose binding domain fused or linked ("fused or linked" = covalently coupled) to one or more biological or chemical entity ranging in size from hundreds to several millions of Daltons. Generally the biological entity is one or more second protein. The second protein can be another PBD, such as a cellulose binding domain or a starch binding domain or a functionalized PBD, such as a PBD bound to a hydrophobic group or an enzyme, particularly an enzyme which can improve and/or accelerate processing of the polysaccharide structures and/or the characteristics of the polysaccharide containing

end product such as a lipase or a laccase. Alternatively, the second protein can be a non-PBD protein such as a protein A-antibody complex.

For preparing a modified polysaccharide-containing material, a polysaccharide structure is contacted during or after processing into a polysaccharide material with a sufficient amount of a biological crosslinker under conditions and for a time sufficient to modify one or more characteristic of a polysaccharide material containing the polysaccharide structure. As an example, instead of or in conjunction with the traditional sizing step during processing of cellulose fibers into paper or cotton into yarn, the PBD fusion proteins can be used to aggregate polysaccharide structures and/or crosslink polysaccharide structures such as polysaccharide fibers and filaments so as to increase the wet strength of the structures themselves during processing and/or the polysaccharide containing material produced. Preferably the treatment with PBD fusion proteins is in lieu of the traditional sizing step. However, in some applications it can be useful to combine the two procedures, for example, with a starch size and PBD fusion proteins containing starch binding proteins to crosslink the starch and the fibers. In addition, PBDs comprising functional moieties can be used to functionalize a polysaccharide containing material, such as a cellulose matrix, with for example a hydrophobic moiety such as a fatty acid derivative or a hydrophobic amino acid sequence to decrease the wetability of a polysaccharide containing material such as paper.

The subject invention offers several advantages over existing methods of treating polysaccharide structures as for example are used in commercial paper and textile processes. By treating a suitable polysaccharide containing material, such as cellulose fibers, with a biological crosslinker, a product with improved mechanical properties (for example, increased strength and durability) as compared with untreated materials and/or materials treated using enhancing materials other than PBD fusion proteins can be obtained. In addition, in the manufacture of fluting paper, the PBD reagent can be applied in either the forming stage or before or after the sizing stage to increase the wet strength of the paper. If applied in the forming stage, it provides sufficient wet strength so that the sizing step can be eliminated. This not only saves time, but it also significantly lowers the cost of preparing the paper, because about one third of the machine used to process the paper can be eliminated. The use of biological crosslinkers as opposed to chemical crosslinkers and hydrophobic materials also improves the recyclability of paper products made using this process.

Another advantage of the subject invention is that in the forming step of paper making, many fine fibers are lost because they pass through the forming fabric. The PBD reagent maintains them in the paper slurry, resulting in a better recovery of raw materials. Additionally, in the final processing step of producing corrugated

containers, an alkaline glue is used to bind the fluting paper to the wallboard. PBD molecules are eluted by strong alkaline conditions, which enhances the ability of the alkaline glue to penetrate the paper.

The multimeric PBD fusion proteins of the subject invention have two basic building blocks, a PBD and a second protein, wherein the second protein may or may not be a PBD. A PBD can be a protein or a peptide that comprises an amino acid sequence that binds to a polysaccharide such as cellulose or a polymer which contains the basic structural units of the polysaccharide substrate to which the PBD binds, including either backbone sugars and/or terminal sugars and sugars themselves, including monosaccharides and disaccharides. Included within the definition of a PBD are mutants, variants and the like of naturally occurring PBDs, the only requirement being that they bind to a polysaccharide containing substrate. PBDs can bind to a substrate polysaccharide either reversibly or irreversibly, and the substrate can be natural or synthetic. A PBD fusion protein can be a protein molecule having multiple polysaccharide binding domains that may be derived from the same or different polysaccharidases or scaffolding proteins and that may bind to the same or different polysaccharides. When multiple PBDs are present, they preferably occupy separate domains within the PBD fusion protein, and may function independently of each other. The term CBD refers to either a domain obtainable from a native protein which is involved in cellulose binding, or to an isolated amino acid sequence or fragment of the native protein which itself binds to cellulose (*see*, for example, Goldstein *et al.* (1993) *J. Bacteriol.* 175:5762-5768 and Gilkes *et al.* (1988) *J. Biol. Chem.* 263:10401-10407, the contents of both of which are incorporated herein by reference). PBDs and CBDs can be natural, synthetic, or partially synthetic.

The polysaccharide binding domains, including both catalytically competent and incompetent polysaccharidases comprising polysaccharide binding domains, can be obtained by any of a variety of techniques, including biochemical and/or genetic engineering techniques. Thus, they can be obtained by proteolysis (see, for example, Gilkes *et al.*, *J. Biol Chem* (1988) 213: 10401-10407) or by gene manipulation using techniques known to those skilled in the art, such as random mutation, site-directed mutagenesis or DNA shuffling. Using site-directed mutagenesis, specific amino acids relating to the catalytic activity of the polysaccharidase can be mutagenized and replaced by amino acids that inhibit or block catalytic activity, but do not interfere with the binding of polysaccharide. For example, in CenA, aspartate at position 283 could be replaced. Such an approach effectively generates an amino acid sequence quite similar to the original polysaccharidase sequence, but the functional domain containing the catalytic activity is rendered incompetent by mutagenesis or biochemical modification; only the binding domain remains functional. One or more

predetermined amino acid residues may be substituted, inserted in, or deleted from the amino acid sequence of various PBDs to provide variant or mutated PBDs. Amino acid substitutions in a PBD protein or polypeptide sequence can be made in a rational manner based, for example, on similarity or differences in polarity, charge, hydrophobicity, hydrophilicity, and the like of targeted amino acid residue(s). Characteristics such as polarity and hydrophobicity of all amino acids commonly found in proteins are well known in the art, as are techniques for specifically changing (mutating) amino acid sequences. The resulting variant or mutated PBDs are deemed to be within the scope of the instant invention. Substitutions, insertions and/or deletions can be made to provide a variant PBD having more desirable attributes, for example, for cross-linking or functionalization of particular polysaccharide-containing materials.

Amino acid sequences corresponding only to the polysaccharide binding domain can be used rather than the entire polysaccharidase sequence with specific mutations or modifications. In this case, PBD is obtained by cleaving the polysaccharidase into functional domains. For example, an isolated polysaccharidase is subjected to protease treatment that cleaves the protein into two or more fragments consisting of functional domains. On occasion, the polysaccharidase contains a specific protease site. For example, *C. fini* endoglucanase A (CenA) contains a PT box that is cleaved by conformation-specific *C. fini* protease. The products of that reaction are a polysaccharide binding domain with a PT sequence and a polysaccharidase catalytic domain. If the polysaccharidase is not cleaved by highly sequence specific proteases it will be subject to less specific proteases, and the active fragments can be isolated by chromatography and other peptide purification techniques known to those skilled in the art.

Other techniques that can be used to obtain a binding domain include use of amino acid sequence information to generate probes for the cloning of DNA sequences encoding polysaccharidases or polysaccharide binding proteins. These cloned sequences can be used to generate deletion mutants encoding only the polysaccharide binding domains. Conversely, if the cDNA sequence of a polysaccharidase or polysaccharide binding protein is known, then a DNA sequence can be specifically constructed that corresponds to the polysaccharide binding domain by using biochemical, amino acid, and DNA sequence data to predict the location of the polysaccharide binding domain based on sequences homologies to other polysaccharidases. The techniques used in isolating polysaccharidase genes and polysaccharide binding proteins are known in the art, including synthesis, isolation from genomic DNA, preparation from cDNA or combinations thereof. Other techniques that can be used to obtain polysaccharide binding domains include gene

fusion, phage display, DNA shuffling and random or site specific mutagenesis. Various techniques for manipulation of genes are well known, and include restriction, digestion, resection, ligation, *in vitro* mutagenesis, primer repair, employing linkers and adapters, and the like (*see* Molecular Cloning: A Laboratory Manual 2nd edition Sambrook *et al.* (eds.), Cold Spring Harbor Laboratory Press, NY, (1989), which is incorporated herein by reference). The nucleic acid encoding a PBD protein of the invention may be obtained from isolated and purified RNA or by genomic cloning. Either cDNA or genomic libraries may be prepared using techniques well known in the art, and may be screened for a particular PBD ennucleotide sequence with probes that are substantially complementary to any portion of the coding sequence. Alternatively, cDNA or genomic DNA may be used as templates for PCR cloning using suitable oligonucleotide primers. Full length clones, i.e., those containing the entire coding sequence of the desired PBD protein may be selected for constructing expression vectors, or overlapping cDNAs can be ligated together to form a complete coding sequence or desired portion thereof, such as the binding domain. Alternatively, DNAs that encode a PBD can be synthesized, in whole or in part, by chemical synthesis using solid-phase techniques well known in the art.

The PBP can be obtained from a variety of sources, including enzymes which bind to oligosaccharides which find use in the subject invention. In Table 5 below are listed those binding domains which bind to one or more soluble/insoluble polysaccharides including all binding domains with affinity for soluble glucans α, β, and/or mixed linkages. The N1 cellulose-binding domain from endoglucanase CenC of *C. fimi* binds to soluble cellosaccharides and one of a small set of proteins which are known to bind any soluble polysaccharides. Also, listed in Tables 1 to 4 are examples of proteins containing putative β-1,3-glucan-binding domains (Table 1); proteins containing *Streptococcal* glucan-binding repeats (Cpl superfamily) (Table 2); enzymes with chitin-binding domains (Table 3), and starch-binding domains (Table 4). Scaffolding proteins which include a cellulose binding domain protein such as that produced by *Clostridium cellulovorans* (Shoseyov *et al.*, PCT/US94/04132) can also be used for preparing a PBP. Several fungi, including *Trichodenma* species and others, also produce polysacchanrdases from which PBP can be isolated.

Table 1

Overview of proteins containing putative β-1,3 glucan-binding domains

Source (strain)	Protein	accession No.	Ref.[1]
Type I			
B. circulans (WL-12)	GLCA1	P23903/M34503/JQ0420	1
B. circulans (IAM 1165)	BglH	JN0772/D17519/S67033	2
Type II			
Actinomadura sp. (FC7)	XynII	U08894	3
Arthrobacter sp. (YCWD3)	GLCI	D23668	9
O. xanthineolytica	GLC	P22222/M60826/A39094	4
R. faecitabidus (YLM-50)	RP I	Q05308/A45053/D10753	5a,b
R. communis	Ricin	A12892	6
S. lividans (1326)	XlnA	P26514/M64551/JS07986	7
T. tridentatus	FactorGa	D16622	8

B. : *Bacillus, O.* : *Oerskovia, R. faecitabidus* : *Rarobacter faecitabidus, R. communis*: *Ricinus communis, S.* : *Streptomyces, T.* : *Tachypleus* (Horseshoe Crab)

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Table 2

Overview of proteins containing Streptococcal glucan-binding repeats (Cpl superfamily)

Source	Protein	Accession No.	Ref.[2]
S. downei (sobrinus) (0MZ176)	GTF-I	D13858	1
S. downei (sobrinus) (MFe28)	GTF-I	P11001/M17391	2
S. downei (sobrinus) (MFe28)	GTF-S	P29336/M30943/A41483	3
S. downei (sobrinus) (6715)	GTF-I	P27470/D90216/A38175	4
S. downei (sobrinus)	DEI	L34406	5
S. mutants (Ingbritt)	GBP	M30945/A37184	6
S. mutants (GS-5)	GTF-B	A33128	7
S. mutants (GS-5)	GTF-B	P08987/M17361/B33135	8
S. mutants	GTF-B[3'-ORF]	P05427/C33135	8
S. mutants (GS-5)	GTF-C	P13470/M17361/M22054	9

S. mutants (GS-5)	GTF-C	not available	10
S. mutants (GS-5)	GTF-D	M29296/A45866	11
S. salivarius	GTF-J	A44811/S22726/S28809 Z11873/M64111	12
S. salivarius	GTF-K	S22737/S22727/Z11872	13
S. salivarius (ATCC25975)	GTF-L	L35495	14
S. salivarius (ATCC25975)	GTF-M	L35928	14
S. pneumoniae R6	LytA	P06653/A25634/M13812	15
S. pneumoniae	PspA	A41971/M74122	16
Phage HB-3	HBL	P32762/M34652	17
Phage Cp-1	CPL-1	P15057/J03586/A31086	18
Phage Cp-9	CPL-9	P19386/M34780/JQ0438	19
Phage EJ-1	EJL	A42936	20
C. difficile (VPI 10463)	ToxA	P16154/A37052/M30307 X51797/S08638	21
C. difficile (BARTS W1)	ToxA	A60991/X17194	22
C. difficile (VPI 10463)	ToxB	P18177/X53138/X60984 S10317	23,24
C. difficile (1470)	ToxB	S44271/Z23277	25,26
C. novyi	a-toxin	S44272/Z23280	27
C. novyi	a-toxin	Z48636	28
C. acetobutylicum (NCIB8052)	CspA	S49255/Z37723	29
C. acetobutylicum (NCIB8052)	CspB	Z50008	30
C. acetobutylicum (NCIB8052)	CspC	Z50033	30
C. acetobutylicum (NCIB8052)	CspD	Z50009	30

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New PBPs with interesting binding characteristics and specificities can be identified and screened for in a of ways including spectroscopic (titration) methods such as: NMR spectroscopy (Zhu *et al.* (1995) *Biochemistry* 34:, Gehring *et al.* (1991) *Biochemistry* 30:5524-5531), UV difference spectroscopy (Beishaw *et al.* (1993) *Eur. J. Biochem.* 211:717-724), fluorescence (titration) spectroscopy (Miller *et al.* (1983) *J. Biol. Chem.* 258:13665-13672), UV or fluorescence stopped flow analysis (De Boeck *et al.* (1985) *Eur. J. Biochem.* 149:141-415), affinity methods such as affinity electrophoresis (Mimura *et al.* (1992) *J. Chronmatography* 597:345-350) or affinity chromatography on immobilized mono or oligosaccharides, precipitation or agglutination analysis including turbidimetric or nephelometric analysis (Knibbs *et al.* (1993) *J. Biol. Chem.* 14940-14947), competitive inhibition assays (with or without quantitative IC50 determination) and various physical or physico-chemical methods including differential scanning or isothermal titration calorimetry (Sigurskjold *et al.* (1992) *J. Biol. Chem.* 267:8371-8376; Sigurskjold *et al.* (1994) *Eur. J. Biol.* 225:133-141) or comparative protein stability assays (melts) in the absence or presence of oligosaccharides using thermal CD or fluorescence spectroscopy.

Generally, the Ka for binding of the PBP to oligosaccharide is at least in the range of weak antibody-antigen extractions, i.e., 10^{-3}, preferably 10^{-4}, most preferably 10^{-6}. If the binding of the PBP to the oligosaccharide is exothermic or endothermic, then binding increases or decreases, respectively, at lower temperatures, providing a means for temperature modulation during polysaccharide structure processing.

Table 3
Overview of enzymes with chitin-binding domains

Source (strain)	Enzyme	Accession No.	Ref.[3]
Bacterial enzymes			
<u>Type I</u>			
Aeromonas sp. (No10S-24)	Chi	D31818	1
Bacillus circulans (WL-12)	ChiA1	P20533/M57601/A38368	2
Bacillus circulans (WL-12)	ChiD	P27050/D10594	3
Janthinobacterium lividum	Chi69	U07025	4

Streptomyces griseus	Protease C	A53669	5
Type II			
Aeromonas cavia (K1)	Chi	U09139	6
Alteromonas sp (0-7)	Chi85	A40633/P32823/D13762	7
Autographa californica (C6)	NPH-128[a]	P41684/L22858	8
Serratia marcescens	ChiA	A25090/X03657/L01455/P07254	9
Type III			
Rhizopus oligosporus (IFO8631)	Chi1	P29026/A47022/D10157/S27418	10
Rhizopus oligosporus (IFO8631)	Chi2	P29027/B47022/D10158/S27419	10
Saccharomyces cerevisiae	Chi	S50371/U17243	11
Saccharomyces cerevisiae Chi1 (DBY939)		P29028/M74069	12
Saccharomyces cerevisiae Chi2 (DBY918)		P29029/M7407/B41035	12
Plant enzymes			
Hevein superfamily			
Allium sativum	Chi	M94105	13
Amaranthus caudatus	AMP-1[b]	P27275/A40240	14, 15
Amaranthus caudatus	AMP-2[b]	S37381/A40240	14, 15
Arabidopsis thaliana (cv. colombia)	ChiB	P19171/M38240/B45511	16
Arabidopsis thaliana	PHP[c]	U01880	17
Brassica napus	Chi	U21848	18
Brassica napus	Chi2	Q09023/M95835	19
Hevea brasiliensis	Hev1[d]	P02877/M36986/A03770/A38288	20, 21
Hordeum vulgare	Chi33	L34211	22
Lycopersicon esculentum	Chi9	Q05538/Z15140/S37344	23
Nicotiana tabacum	CBP20[e]	S72424	24
Nicotiana tabacum	Chi	A21091	25
Nicotiana tabacum (cv. Havana)	Chi	A29074/M15173/S20981/S19855	26
Nicotiana tabacum (FB7-1)	Chi	JQ0993/S0828	27
Nicotiana tabacum (cv. Samsun)	Chi	A16119	28
Nicotiana tabacum (cv. Havana)	Chi	P08252/X16939/S08627	27
Nicotiana tabacum (cv. BY4)	Chi	P24091/X51599/X64519//S13322	26,27,29
Nicotiana tabacum (cv. Havana)	Chi	P29059/X64518/S20982	26
Oryza sativum (IR36)	ChiA	L37289	30
Oryza sativum	ChiB	JC2253/S42829/Z29962	31
Oryza sativum	Chi	S39979/S40414/X56787	32
Oryza sativum (cv. Japonicum)	Chi	X56063	33
Oryza sativum (cv. Japonicum)	Chi1	P24626/X54367/S14948	34
Oryza sativum	Chi2	P25765/S15997	35
Oryza sativum (cv. Japonicum)	Chi3	D16223	
Oryza sativum	ChiA	JC2252/S42828	30
Oryza sativum	Chi1	D16221	32
Oryza sativum (IR58)	Chi	U02286	36
Oryza sativum	Chi	X87109	37
Pisum sativum (cv. Birte)	Chi	P36907/X63899	38
Pisum sativum (cv. Alcan)	Chi2	L37876	39
Populus trichocarpa	Chi	S18750/S18751/X59995/P29032	40
Populus trichocarpa (H11-11)	Chi	U01660	41

Phaseolus vulgaris (cv. Saxa)	Chi	A24215/S43926/Jq0965/P36361	42
Phaseolus vulgaris (cv. Saxa)	Chi	P06215/M13968/M19052/A25898	43,44,45
Sambucus nigra	PR-3[f]	Z46948	46
Secale cereale	Chi	JC2071	47
Solanum tuberosum	ChiB1	U02605	48
Solanum tuberosum	ChiB2	U02606	48
Solanum tuberosum	ChiB3	U02607/S43317	48
Solanum tuberosum	ChiB4	U02608	48
Solanum tuberosum (cv. Maris Piper)	WIN-1[g]	P09761/X13497/S04926	49
Solanum tuberosum (cv. Maris Piper)	WIN-2[g]	P09762/X13497/S04927	49
Triticum aestivum	Chi	S38670/X76041	50
Triticum aestivum	WGA-1[h]	P10968/M25536/S09623/S07289	51,52
Triticum aestivum	WGA-2[h]	P02876/M25537/S09624	51,53
Triticum aestivum	WGA-3	P10969/J02961/S10045/A28401	54
Ulmus americana (NPS3-487)	Chi	L22032	55
Urtica dioica	AGL[i]	M87302	56
Vigna unguiculata (cv. Red caloona)	Chi1	X88800	57

[a]NHP : nuclear polyhedrosis virus endochitinase like sequence; Chi : chitinase, [b]anti-microbial peptide, [c]pre-hevein like protein, [d]hevein, [e]chitin-binding protein, [f]pathogenesis related protein, [g]wound-induced protein, [h]wheat germ agglutinin, [i]agglutinin (lectin).

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Table 4

Overview of enzymes containing starch-binding domains

Source (strain)	Enzyme	Accession No.	Ref.[4]
A. awarori (var. *kawachi*)	AMYG	P23176/D00427/JT0479	1, 2
A. niger (T21)	AMYG	S73370	3
A. niger -A. awamori	AMYG1/G2	P04064/A90986/A29166/X00712/ X00548	4,5,6
		K02465	7,8,9
A. oryzae	AMYG (GLAA)	P36914/JQ1346/D01035/S75274/ D01108	10, 11
A. Shirousamii	AMYG (GLA)	P22832/JQ0607/D10460	12
Bacillus sp. (B1018)	AMY[a]	P17692/M33302/D90112/S09196	13
Bacillus sp. (TS-23)	α-AMY	U22045	14
Bacillus sp. (1-1)	CGT	P31746/S26399	15
Bacillus sp. (6.63)	CGT	P31747/X66106/S21532	16
Bacillus sp. (17-1)	CGT	P30921/M28053/A37208	17
Bacillus sp. (38-2)	CGT	P09121/M19880/D00129/S24193	18, 19
Bacillus sp. (1011)	CGT	P05618/A26678/M17366	20
Bacillus sp. (DSM5850)	CGT	A18991	21
Bacillus sp. (KC 201)	CGT	D13068	15, 22
B. cereus (SPOII)	β-AMY	A48961/P36924/S54911	23
B. circulans (8)	CGT	P30920/X68326/S23674	24
B. circulans (251)	CGT	X78145	25
B. Licheniformis	CGTA	P14014/X15752/S15920	26
B. macerans (IFO 3490)	CGTM (CDG1)	P04830/X5904/S31281	27
B. macerans (IAM 1243)	CGT	M12777	28

B. macerans	CGT (CDG2)	P31835/S26589	29
B. ohbensis	CGT	P27036/D90243	30
B. stearothermophilus	AMYM[b]	P19531/M36539/S28784	31
B. stearothermophilus (NO2)	CGT	P31797/X59042/S26588/X59043/ X59404/S31284	32
C. rolfsii (AHU 9627)	AMYG2	D49448	33
D. discoideum	ORF	S15693/X51947	34
H. grisea (var. *thermoidea*)	GLA1	M89475	35
H. resinae (ATCC20495)	GAMP	Q03045/X68143/X67708/S31422/ S33908	36-38
K. pneumoniae (M5A1)	CGT	P08704/M15264/A29023	39
N. crassa (74-OR23-1A)	GLA-1	P14804/X67291/S13711/S13710/ S36364	40, 41
P. saccharophila (IAM1504)	MTA[c]	P22963/X16732/S05667	42
Pseudomonas sp. (KO-8940)	AMF-1[d]	D10769/JS0631/D01143	43
P. stutzeri (MO-19)	AMYP[c]	P13507/M24516/A32803	44
S. griseus (IMRU 3570)	AMY	P30270/X57568/S14063	45
S. limosus (S.albidoflavus) AML		P09794/ M18244/B28391	46
S. violaceus (S. venezuela) AML (ATCC15068)		P22998/M25263/JS0101	47
Th. curvata (CCM 3352)	TAM[e]	P29750/X59159/JH0638	48
Th. thermosulfurogenes[f] (DSM3896/EM1)	AMYA	P26827/X54654/X54982/ S17298/S37706	49
Th. thermosulfurogenes (ATCC 33743)	AMYB	P19584/M22471/A31389	50

[a]Raw-starch digesting amylase, [b]Maltogenic α-amylase, [c]Maltotetraose-forming amylase (1,4-α-maltotetrahydrolase), [d]Maltopentaose-forming amylase, [e]thermostable α-amylase, [f]formerly *Clostridium thermosulfurogenes*. AMYG, GAM and GLA: glucoamylase, AMY or AML: alpha-amylase, CGT: β-cyclodextrin glycosyltransferase or cyclomaltodextrin glucanotransferase, ORF: open reading frame. *A.*: *Aspergillus*, *B.*: *Bacillus*, *C.*: *Corticium*, *D.*: *Dictiostelium*, *H. grisea*: *Humicola grisea*, *H. resinea*: *Hormoconis resinae* (*Amorphotheca resinae*), *K.*: *Klebsiella*, *N.*: *Neurospora*, *S.*: *Streptomyces*, *Th. curvata*: *Thermomonospora curvata*, *Th.*: *Thermoanaerobacter*.

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Table 5
Sources of polysaccharide binding domains

Binding Domain	Proteins Where Binding Domain is Found
Cellulose Binding Domains[1]	β-glucanases (avicelases, CMCases, cellodextrinases) exoglucanses or cellobiohydrolases cellulose binding proteins xylanases mixed xylanases/glucanases esterases chitinases β-1,3-glucanases β-1,3-(β-1,4)-glucanases (β-)mannanases β-glucosidases/galactosidases cellulose synthases (unconfirmed)
Starch/Maltodextrin Binding Domains	α-amylases[2,3] β-amylases[4,5]

	pullulanases
	glucoamylases[6,7]
	cyclodextrin glucotransferases[8-10]
	(cyclomaltodextrin glucanotransferases)
	maltodextrin binding proteins[11]
Dextran Binding Domains	*(Streptococcal)* glycosyl transferases[12]
	dextran sucrases (unconfirmed)
	Clostridial toxins[13,14]
	glucoamylases[6]
	dextran binding proteins
β-Glucan Binding Domains	β-1,3-glucanases[15,16]
	β-1,3-(β-1,4)-glucanases (unconfirmed)[17]
	β-1,3-glucan binding protein
Chitin Binding Domains	chitinases
	chitobiases
	chitin binding proteins
	(see *also* cellulose binding domains)
	Heivein

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Numerous CBDs are known and are classified into at least 12 Families, any of which can serve as a source of CBDs depending upon the intended use of the CBD. Family I contains only CBDs of fungal enzymes. The vast majority of CBDs in the remaining 11 Families are of bacterial origin. The best understood CBDs are those belonging to Families I, II, III, and IV, the CBDs of which are on average 36, 105, 150, and 150 amino acids in length, respectively. Some CBDs of Families I, II, III, and IV have been characterized as comprising a plurality of anti-parallel β-sheets folded into jelly rolls CBDs of families I, II, and III bind to both amorphous and crystalline cellulose, whereas CBDs of family IV bind to amorphous cellulose, but not to crystalline cellulose. Only CBDs of family IV bind to solublcellulose derivatives and cellooligosacharides. CBDs that bind to crystalline cellulose and chitin do so with similar affinities, having binding constants in the micromolar range. Family I CBDs

bind reversibly to cellulose, whereas Family II and III CBDs appear to bind irreversibly under non-denaturing conditions. Preferred CBDs include those obtainable from strains belonging to the species of *Cellulomonas fimi, Trichoderma reesei* and *M. Bispora* (N.R. Gilkes *et al.*, (1988) *J. Biol. Chem.* 263: 10401-10407; N.R Gilkes *et al.*, (1991), *Microbiol. Rev.* 55: 303-315); cellulase genes from *Cellulomonas fimi* (Whittle *et al.* (1982) *Gene 17: 139-145*; Gilkes *et al.* (1984) *J. Gen. Microbiol.* 130: 1377-1384); an exoglucanase (Cex) and an endoglucanase (CenA) from *C. fimi* and sequences of their genes, *cex* and *cenA* (Wong *et al.* (1986) *Gene* 44: 315-324; O'Neill *et al.* (1986) *Gene* 44: 325-330); a 17 KD (peptide) CBD derived from *Clostridium cellulovorans* described by Shoseyov *et al.* (1992) (*Proc. Natl. Acad. Sci.* 89: 3483-3487). Recombinant forms of this CBD exhibit strong affinity for cellulose and chitin (Goldstein *et al.* (1993) *J. Bacteriol.* 175:5762-5768).

The PBD protein also can be prepared by transforming into a host cell a DNA construct comprising DNA encoding at least a functional portion of the polysaccharide binding region of a polysaccharidase or a polysaccharide binding protein. The PBD DNA sequence can be expressed in a host cell, either a eukaryotic or a prokaryotic cell. Expressed and isolated PBD's then can be conjugated to other PBDs and/or one or more functionating protein.

In any of these cases, the isolated polysaccharide binding domain generally is sufficiently pure to exclude catalytic polysaccharidase activity unless this is a desired feature of the intended fusion protein. Preferably, the catalytic activity of such preparation is less than that of crude extracts from cells expressing the polysaccharidase. More preferably, the catalytic activity will reflect a stoichiometry of less than 1 functional catalytic domain per 1000 functional binding domains. To test the activity of a desired expression product, the binding activity of a PBD can be determined, for example, by binding to microcrystalline cellulose such as Avicel (microcyrstalline cellulose) and showing that the putative binding domain is removed from solution. A polypeptide having the desired activity is readily isolated in highly purified form from the cellulose. Binding to Avicel has been used for purification of both native (Gilkes *et al., J. Biol. Chem.* (1984) *259*:10455-10459) and recombinant cellulases (Owolabi *et al., Appl. Environ. Microbiol.* (1988) *54*:518-523).

The second basic building block of the multimeric PBD fusion protein is a protein which can be a second PBD which can be the same as, or different from, the PBD which is the first building block. Thus the multimeric fusion protein can be a dimeric PBD fusion protein encoded by a pair of nucleotide sequences, each encoding a PBD, ligated in frame as is well known in the art (*see*, for example, U.S. PAT. NO. 5, 856,201 and U.S. PAT. NO. 5,837,814 both to Shoseyov, *et al.*, both of which are incorporated by reference herein in their entirety). Shown in Fig. 5A is an example of

a PBD fusion protein in which both the first and the second proteins are CBDs, thus forming a dimeric CBD, wherein the CBDs may be the same (homodimeric-CBDs) or different (heterodimeric-CBDs)). Shown in Fig. 5B is the use of the cellulose cross-linking protein of Fig. 5A, wherein one cellulose binding domain is bound to a first polymeric structural unit, and a second cellulose binding domain is bound to a second polymeric structural unit. Fig. 6 schematically represents a generic CBD coupler unit including a pair of CBDs linked via a linker unit.

Alternatively, the second building block can be a PBD that optionally includes one or more functionating group. By a functionating group is intended a functional group that can modify one or more property of a polysaccharide containing material. Generally the functionating group is a protein or a peptide such as a silicon binding peptide, polymer binding peptide or a metal binding peptide (Ljungquist *et al,* (1989) *Eur. J. Biochem.* 86: 563-569; Spanner *et al*, ((1995) *Bone* 17: 161-165; Slice *et al* (1990) *J. Biol. Chem.* 265: 256-263; Pessi *et al* (1993) *Nature* 362: 367-369). Other examples of functionating polypeptides include a starch binding domain which provides a means for crosslinking of polysaccharide fibers and starch molecules; the starch can be an endogenous component of the fibers, or can be applied as a size. Starch binding domains can be obtained, for example, from *Aspergillus* glucoamylase (Chen *et al.* (1991), *Gene* 99:121-126). Likewise, polysaccharide and gluten molecules can be crosslinked by using as a second polypeptide a matrix protein such as high molecular weight glutinin (HMWG). For particular applications the functionating group also can include chemical groups such as one or more thiol group, chromophore, dye, a reactive group such as an aldehyde, a maleimide, a hydrazide, an epoxy, a carbodiimide, or a photo reactive group such as phenyl azide bound to a PBD. Methods for conjugating various chemical entities to a PBD are described in U.S. PAT. NO. 5,962,289, which is incorporated herein by reference herein in its entirety.

The first building block of the PBD fusion protein can optionally be linked to the second building block via a linker unit. The linker unit of a PBD coupler unit can include various natural or synthetic molecules, including biological polymers such as a protein, a polypeptide, or a polysaccharide, and synthetic polymers such as acrylic polymers and the matrix protein High Molecular Weight Glutinin. Examples of peptide or protein components of a linker unit include JUN protein and FOS protein (*see*, for example, Gentz *et al.*, (1989) *Science* 243: 1695-1699); starch binding domain (SBD) (*see*, for example, Chen *et al.* (1991) *Gene* 99: 121-126), and S-peptide or S-protein (*see*, for example, Kim *et al.* (1993) *Protein Science* 2: 348-356). The first and the second polypeptides (or multiple first and/or second polypeptides) in the fusion protein can be joined directly via a peptide bond, or a larger linker unit,

depending in part upon the intended use of the fusion protein. Fig. 8 shows schematically a CBD coupler unit (designated CU in the figure) having a first CBD, a second CBD, and a linker unit (LU) linking the first and second CBDs. Although the first and second CBDs are depicted in Fig. 8 as being terminal, and as being located at opposite poles of the linker unit, other numbers and arrangements of CBDs and linker unit(s) are contemplated and are within the scope of the invention. The linker unit can be attached to each PBD of a PBD coupler unit by one or a combination of various means, including covalent bonding, ionic bonding, hydrophobic bonding, hydrogen bonding, protein translation, and protein expression.

A polysaccharide component of a linker unit can be a polysaccharide which is not bound, or bound with low affinity, by a PBD. An example of such a polysaccharide is starch. In addition, a linker unit of a PBD coupler unit can be one or more polysaccharide binding domains other than a PBD. As an example, Fig. 7A shows a CBD coupler unit having a pair of terminal CBDs linked by a coupler unit which includes a first starch binding domain coupled to a first CBD, a second starch binding domain coupled to a second CBD, and a starch moiety coupled to both the first starch binding domain and the second starch binding domain.

A linker unit of a PBD coupler unit also can include one or more PBDs. As an example, Fig. 7B shows a CBD coupler unit having a pair of terminal CBDs linked by a coupler unit which includes a plurality of CBDs, wherein each CBD of the linker unit is coupled to an adjacent CBD via a JUN/FOS bridge (see, for example, Gentz *et al.*, (1989) *Science* 243: 1695-1699). Fig. 7C shows a CBD coupler unit having a pair of terminal CBDs linked by a coupler unit which includes a peptide or protein moiety which does not bind, or binds with only low affinity, to cellulose or related polymers. A peptide or protein component of a coupler unit, for example, a linker protein, may vary in size from a few hundred Daltons to more than 1 MegaDaltons. As an example, the linker unit represented in Fig. 7C can be a short peptide of a few amino acids, or a relatively large linker protein, such as HMWG.

The multimeric PBD fusion protein can be made chemically or recombinantly. For example, the polysaccharide binding region or multiples thereof can be produced on its own, purified and then chemically linked to a second protein with or without a functionating group using techniques known to those skilled in the art. Methods of protein conjugation include *in vitro* conjugation chemical reactions to modify the polysaccharide binding domain which can be carried out while the domain is either bound to a polysaccharide matrix or free from the polysaccharide matrix. Examples include the use of gluteraldehyde conjugation as described by Reichlin in *Methods of Enzymology* (1980) 70:159-165. When the polysaccharide binding domain is bound to the matrix, it offers the advantage of protecting the site that actually binds to the

matrix while leaving other residues to react with the second moiety, either a second PBD or a functionating protein. If bonding of the chemical moiety to the polysaccharide binding domain results in a diminished capacity to bind the polysaccharide substrate, a reaction procedure requiring the presence of the polysaccharide matrix is preferred to retain the binding characteristics of the domain.

Alternatively the multimeric PBD fusion protein can be made recombinantly. To make a PBD fusion protein recombinantly, nucleotide sequences encoding the components of the PBD fusion protein are used to construct recombinant expression vectors capable of expressing PBD fusion proteins. In general, a nucleic acid construct is capable of expressing a protein if it contains nucleotide sequences containing transcriptional and translational regulatory information which are operably linked to nucleotide coding sequences for the protein. "Operably linked" refers to a linkage in which the regulatory DNA sequences and the DNA sequence to be expressed are connected in such a way as to permit transcription and translation. The polysaccharide binding domain encoding fragment and the DNA encoding the second polysaccharide binding domain or functionating polypeptide are ligated so that the nucleic acid encoding the PBD is joined to the nucleic acid encoding the second protein such that the combined open reading frame of the PBD and the second protein is intact, allowing translation of the entire PBD fusion protein to occur. If the PBD fusion protein has a protein coupler unit, the nucleotide sequences are operably inserted into the expression construct, between the PBD encoding sequence and the sequence encoding the second protein. The resulting ligated DNA can then be manipulated in a variety of ways to provide for expression.

Vectors for both nucleic acid amplification and for nucleic acid expression are well known in the art. Selection of an appropriate vector depends on various parameters including, the intended function (e.g., amplification or expression), the size of the DNA insert, and the particular host cell to be transformed with the vector. Various expression vector/host systems may be utilized by the skilled artisan for the recombinant expression of PBD proteins and PBD fusion proteins. Such systems include microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the desired PBD coding sequence; yeast transformed with recombinant yeast expression vectors containing the desired PBD coding sequence; insect cell systems infected with recombinant virus expression vectors (for example, baculovirus) containing the desired PBD coding sequence; plant cell systems infected with recombinant virus expression vectors (for example, cauliflower mosaic virus (CaMV); tobacco mosaic virus, (TMV)) or transformed with recombinant plasmid expression vectors (for example, the Ti plasmid) containing the desired PBD coding sequence; or animal cell

systems infected with recombinant virus expression vectors (for example, adenovirus or vaccinia virus) including cell lines engineered to contain multiple copies of the PBD nucleic acid either stably amplified (for example, CHO/dhfr, CHO/glutamine synthetase) or unstably amplified in double-minute chromosomes (for example, murine cell lines).

Construction of suitable vectors containing one or more of the above listed components and including the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or nucleic acid fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required (see, *Current Protocols in Molecular Biology*, Volumes I-III Ausubel, R. M., ed. (1994)). In order to confirm the correct sequences in DNA constructs (for example, plasmids), ligation mixtures are used to transform *E. coli* strains Xl-1 and DH52 and successful transformants are selected by antibiotic (for example ampicillin) resistance, as appropriate. Plasmids from the transformants are prepared, and analyzed by restriction and/or sequenced (see, for example, Messing *et al.*, *Nucleic Acids Res.* 9: 309 (1981); Maxam *et al.*, *Methods in Enzymology* 65: 499 (1980)). In general, expression vectors are capable of replicating efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of the protein of interest. The expression cassette can be included within a replication system for episomal maintenance in an appropriate cellular host or can be provided without a replication system, where it can become integrated into the host genome.

Once the DNA encoding a PBD fusion protein has been obtained, it is placed in a vector capable of replication in a host cell, or is propagated *in vitro* by means of techniques such as PCR or long PCR. Replicating vectors can include plasmids, phage, viruses, cosmids, artificial chromosomes and the like. Desirable vectors include those useful for mutagenesis of the gene of interest or for expression of the gene of interest in host cells. The technique of long PCR has made *in vitro* propagation of large constructs possible, so that modifications to the gene of interest, such as mutagenesis or addition of expression signals, and propagation of the resulting constructs can occur entirely *in vitro* without the use of a replicating vector or a host cell.

For expression of a PBD fusion protein, functional transcriptional and translational initiation and termination regions are operably linked to the DNA encoding the PBD fusion protein. Expression of the fusion protein coding region can take place *in vitro* or in a host cell. Transcriptional and translational initiation and termination regions are derived from a variety of nonexclusive sources, including the DNA to be expressed, genes known or suspected to be capable of expression in the

desired system, expression vectors, chemical synthesis, or from an endogenous locus in a host cell.

In vitro expression can be accomplished, for example, by placing the coding region for the PBD fusion protein in an expression vector designed for *in vitro* use and adding rabbit reticulocyte lysate and cofactors; labeled amino acids can be incorporated if desired. Such *in vitro* expression vectors may provide some or all of the expression signals necessary in the system used. These methods are well known in the art and the components of the system are commercially available. The reaction mixture can then be assayed directly for the fusion protein, for example by determining its binding activity, or the synthesized fusion protein can be purified and then assayed.

Expression in a host cell can be accomplished in a transient or stable fashion. Transient expression can occur from introduced constructs which contain expression signals functional in the host cell, but which constructs do not replicate and rarely integrate in the host cell, or where the host cell is not proliferating. Transient expression also can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest, although such inducible systems frequently exhibit a low basal level of expression. Stable expression can be achieved by introduction of a construct that can integrate into the host genome or that autonomously replicates in the host cell. Stable expression of the gene of interest can be selected for through the use of a selectable marker located on or transfected with the expression construct, followed by selection for cells expressing the marker. When stable expression results from integration, integration of constructs can occur randomly within the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to target recombination with the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus.

When increased expression of the PBD fusion protein in the source organism is desired, several methods can be employed. Additional genes encoding the PBD fusion protein can be introduced into the host organism. Expression also can be increased, for example, by using a stronger promoter by removing destabilizing sequences from either the mRNA or the encoded protein by deleting that information from the host genome, or by adding stabilizing sequences to the mRNA (U.S. Pat. No. 4,910,141).

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the nucleic acid encoding the polypeptide or protein of interest. Promoters are untranslated sequences which are

located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp of the start codon) and control the transcription and translation of a particular nucleic acid sequence, such as that encoding a PBD fusion protein, to which they are operably linked.

Promoters typically fall into two classes: inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from nucleic acid under their control in response to some change in culture conditions, for example, the presence or absence of a nutrient or a change in temperature. A large number of promoters recognized by a variety of potential host cells are well known in the art. The promoter is operably linked to the nucleic acid encoding the fusion protein by removing the promoter from a source nucleic acid by restriction enzyme digestion and inserting the isolated promoter sequence into a vector together with the coding sequence for the fusion protein. The promoter can be synthetic, semisynthetic, a native (to the host cell) promoter sequence or a heterologous (to the host cell) promoter can be used to direct amplification and/or expression of the fusion protein. Promoters suitable for use with prokaryotic hosts are well known in the art (see, for example, Chang *et al.* (1978) *Nature* 275:615; Goeddel *et al.*(1979) *Nature* 281:544; Goeddel (1980) *Nucleic Acids Res.* 8:4057; EPO Appln. Publ. No. 36,776; and H. de Boer *et al.* (1983) *Proc. Natl. Acad. Sci.* 80: 21-25). The nucleotide sequences of such promoters are generally known, thereby enabling the skilled artisan to operably ligate them to a fusion protein-encoding nucleotide sequence (*see* Siebenlist *et al.*, (1980) *Cell* 20: 269), using linkers or adapters to supply any required restriction sites.

Promoters for use in bacterial systems also contain a Shine-Dalgarno (S.D.) sequence operably linked to the PBD-encoding nucleic acid. Illustrative transcriptional regulatory regions or promoters include, for bacteria, the lac promoter lambda left and right promoters, trp and lac promoters, tac promoter, and the like. The transcriptional regulatory region may additionally include regulatory sequences which allow the time of expression of the fused gene to be modulated, for example the presence or absence of nutrients or expression products in the growth medium, temperature, etc. For example, expression of the fusion gene can be regulated by temperature using a regulatory sequence comprising the bacteriophage lambda PL promoter, the bacteriophage lambda OL operator and a temperature sensitive repressor. Regulation of the promoter is achieved through interaction between the repressor and the operator. Expression vectors used in prokaryotic host cells also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Expression from certain promoters can be elevated in the presence of certain inducers (for example, zinc and cadmium ions for metallothionein promoters). In this manner, expression of the PBD fusion protein can be controlled. The ability to control

expression can be important, for example, if the PBD fusion protein is lethal to a host cell.

Where the host cell is a yeast, transcriptional and translational regions functional in yeast cells are provided, particularly from the host species. The transcriptional initiation regulatory regions can be obtained, for example from genes in the glycolytic pathway, such as alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase (GPD), phosphoglucoisomerase, phosphoglycerate kinase, etc. or regulatable genes such as acid phosphatase, lactase, metallothionein, glucoamylase, etc. Any one of a number of regulatory sequences can be used in a particular situation, depending upon whether constitutive or induced transcription is desired, the particular efficiency of the promoter in conjunction with the open-reading frame of interest, the ability to join a strong promoter with a control region from a different promoter which allows for inducible transcription, ease of construction, and the like. Of particular interest are promoters which are activated in the presence of galactose. Galactose-inducible promoters (GAL1, GAL7, and GAL10) have been extensively utilized for high level and regulated expression of protein in yeast (Lue *et al.*, *Mol. Cell. Biol.* Vol. 7, p. 3446, 1987; Johnston, *Microbiol. Rev.* Vol. 51, p. 458, 1987). Transcription from the GAL promoters is activated by the GAL4 protein, which binds to the promoter region and activates transcription when galactose is present. In the absence of galactose, the antagonist GAL80 binds to GAL4 and prevents GAL4 from activating transcription. Addition of galactose prevents GAL80 from inhibiting activation by GAL4.

Nucleotide sequences surrounding the translational initiation codon ATG have been found to affect expression in yeast cells. If the desired polypeptide is poorly expressed in yeast, the nucleotide sequences of exogenous genes can be modified to include an efficient yeast translation initiation sequence to obtain optimal gene expression. For expression in *Saccharomyces*, this can be done by site-directed mutagenesis of an inefficiently expressed gene by fusing it in-frame to an endogenous *Saccharomyces* gene, preferably a highly expressed gene, such as the lactase gene.

The termination region can be derived from the 3' region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known to and have been found to be satisfactory in a variety of hosts from the same and different genera and species. The termination region usually is selected more as a matter of convenience rather than because of any particular property. Preferably, the termination region is derived from a yeast gene, particularly *Saccharomyces*, *Schizosaccharomyces*, *Candida* or *Kluyveromyces*. The 3' regions of two mammalian genes, χ interferon and α 2 interferon, are also known to function in yeast.

In some instances, it can be desirable to provide for a signal sequence (secretory leader) upstream from and in reading frame with the structural gene, which provides for secretion of the fused gene. Illustrative secretory leaders include the secretory leaders of penicillinase, immunoglobulins, T-cell receptors, outer membrane proteins, and the like. By fusion in proper reading frame the chimeric polypeptide can be secreted into the medium.

Constructs comprising the coding sequences for the fusion protein can be introduced into a host cell by standard techniques. These techniques include transformation, protoplast fusion, lipofection, transfection, transduction, conjugation, infection, biolistic impact, electroporation, microinjection, scraping, or any other method which introduces the gof interest into the host cell. Methods of transformation which are used include lithium acetate transformation (*Methods in Enzymology*, Vol. 194, p. 186-187, 1991). Various methods for genetic transformation of prokaryotic and eukaryotic organisms or cells are well known in the art (*see*, for example, Cohen *et al.* (1972) *Proc. Natl. Acad. Sci.* (USA) 69: 2110; and *Current Protocols in Molecular Biology*, *supra*). Host cells may be transfected, or more preferably transformed, with the above-described expression or cloning vectors of the invention, and the transformed cells may be cultured in conventional nutrient media which may be modified as appropriate for inducing promoters, selecting transformants, or amplifying the gene(s) encoding the desired PBD or PBD fusion protein. By "transformation" is meant the introduction of a nucleic acid into an organism such that the nucleic acid is replicable, either as an extra-chromosomal element or by integration into the genome of the host organism.

The subject host will have at least one copy of the expression construct and may have two or more, depending upon whether the gene is integrated into the genome, amplified, or is present on an extrachromosomal element having multiple copy numbers. Where the subject host is a yeast, four principal types of yeast plasmid vectors can be used: Yeast Integrating plasmids (YIps), Yeast Replicating plasmids (YRps), Yeast Centromere plasmids (YCps), and Yeast Episomal plasmids (YEps). YIps lack a yeast replication origin and must be propagated as integrated elements in the yeast genome. YRps have a chromosomally derived autonomously replicating sequence and are propagated as medium copy number (20 to 40), autonomously replicating, unstably segregating plasmids. YCps have both a replication origin and a centromere sequence and propagate as low copy number (10-20), autonomously replicating, stably segregating plasmids. YEps have an origin of replication from the yeast 2 μ plasmid and are propagated as high copy number, autonomously replicating, irregularly segregating plasmids. The presence of the plasmids in yeast can be ensured by maintaining selection for a marker on the plasmid. Of particular interest are the

yeast vectors pYES2 (a YEp plasmid available from Invitrogen, confers uracil prototrophy and a GAL1 galactose-inducible promoter for expression), pRS425-pG1 (a YEp plasmid obtained from Dr. T. H. Chang, Ass. Professor of Molecular Genetics, Ohio State University, containing a constitutive GPD promoter and conferring leucine prototrophy), and pYX424 (a YEp plasmid having a constitutive TP1 promoter and conferring leucine prototrophy; Alber and Kawasaki (1982). *J. Mol. & Appl. Genetics* 1: 419).

The transformed host cell can be identified by selection for a marker contained on the introduced construct. Alternatively, a separate marker construct may be introduced with the desired construct, as many transformation techniques introduce many DNA molecules into host cells. Typically, transformed hosts are selected for their ability to grow on selective media. Selective media may incorporate an antibiotic or lack a factor necessary for growth of the untransformed host, such as a nutrient or growth factor. An introduced marker gene therefor may confer antibiotic resistance, or encode an essential growth factor or enzyme, and permit growth on selective media when expressed in the transformed host. Selection of a transformed host also can occur when the expressed marker protein can be detected, either directly or indirectly. The marker protein may be expressed alone or as a fusion to another protein. The marker protein can be detected by its enzymatic activity; for example β galactosidase can convert the substrate X-gal to a colored product, and luciferase can convert luciferin to a light-emitting product. The marker protein can be detected by its light-producing or modifying characteristics; for example, the green fluorescent protein (GFP) of *Aequorea victoria* fluoresces when illuminated with blue light. Antibodies can be used to detect the marker protein or a molecular tag on, for example, a protein of interest. Cells expressing the marker protein or tag can be selected, for example, visually, or by techniques such as FACS or panning using antibodies. For selection of yeast transformants, any marker that functions in yeast may be used. Desirably, resistance to kanamycin and the amino glycoside G418 are of interest, as well as ability to grow on media lacking uracil, leucine, lysine or tryptophan.

Once the fused gene has been introduced into an appropriate host, the host can be grown to express the fused gene in conventional nutrient media (modified as appropriate) for inducing promoters, selecting transformants or amplifying genes. Prokaryotic cells used to produce polypeptide or proteins of the instant invention may be cultured in suitable media as described generally in Sambrook *et al.* (1989) *Bacterial Media in Molecular Cloning* (Nolan, C. ed.), Cold Spring Harbor Laboratory Press, NY, pp. A.1-4, which is incorporated herein by reference. Where the product is secreted, the nutrient medium can be collected and the product isolated by binding to a polysaccharide matrix. Where the product is retained in the host cell,

the cells are harvested, lysed and the product isolated and purified by binding to a polysaccharide substrate. To produce an active protein it can be necessary to allow the protein to refold. A host cell strain is chosen that modulates expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. The term "host cell" may be defined as those cells capable of expressing a PBD protein or PBD fusion protein of interest. Host cells can include prokaryotic cells (bacterial) and eukaryotic cells (mammalian, yeast, insect, plant, etc.). Modifications (for example, phosphorylation) and processing (for example, cleavage) of protein products may be important for the function of the protein. Different host cells often have characteristic or specific mechanisms for the post-translational processing of an expressed protein. Appropriate cell lines or host systems may be chosen to ensure the correct modification and processing of the PBD protein or PBD fusion protein expressed. As an example, the recombinant products can be glycosylated or non-glycosylated, having the wild-type or other glycosylation. The amount of glycosylation depends in part upon the sequence of the particular peptide, as well as the organism in which it is produced. Thus expression of the product in *E.coli* cells results in an unglycosylated product, and expression of the product in insect cells generally results in less glycosylation than expression of the product in mammalian cells. Expression in yeast can result in hyperglycosylation. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. In the event that expression is to be performed in a eukaryotic host (for example, plants or mammals), it is preferred that none of the constructs contain potential glycosylation sites.

Production of PBD fusion proteins can be performed in either prokaryotic or eukaryotic host cells. Prokaryotic cells of interest include *Eschericia*, *Bacillus*, *Lactobacillus*, *cyanobacteria* and the like. A prokaryotic cell of particular interest for cloning and expression of PBD fusion proteins is *E. coli* strain BL2(DE3)PLYS. Eukaryotic cells include mammalian cells such as those of lactating animals, avian cells such as of chickens, and other cells amenable to genetic manipulation including insect, fungal, plant and algae cells. The cells may be cultured or formed as part or all of a host organism including an animal. Viruses and bacteriophage also may be used with the cells in the production of PBD fusion proteins, particularly for gene transfer, cellular targeting and selection. Examples of host animals include mice, rats, rabbits, chickens, quail, turkeys, bovines, sheep, pigs, goats, yaks, etc., which are amenable to genetic manipulation and cloning for rapid expansion of the transgene expressing population. For animals, the PBD fusion protein coding sequence can be adapted for expression in target organelles, tissues and body fluids, such as the breast milk of the host animal, through modification of the gene regulatory regions.

Examples of host microorganisms include *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, or other yeast such as *Candida*, *Kluyveromyces* or other fungi, for example, filamentous fungi such as *Aspergillus*, *Neurospora*, *Penicillium*, etc. Desirable characteristics of a host microorganism are, for example, that it is genetically well characterized, and can be used for high level expression of the product using ultra-high density fermentation.

For producing PBD fusion proteins in avian species and cells, such as chickens, turkeys, quail and ducks, gene transfer can be performed by introducing a nucleic acid sequence encoding a PBD fusion protein into the cells following procedures known in the art. If a transgenic animal is desired, pluripotent stem cells of embryos can be provided with a vector carrying a PBD fusion protein encoding transgene and developed into an adult animal (U.S. Pat. No. 5,162,215; Ono *et al.* (1996) *Comparative Biochemistry and Physiology* A 113(3):287-292; WO 9612793; WO 9606160). In most cases, the transgene is modified to express high levels of the PBD fusion protein. The transgene can be modified, for example, by providing transcriptional and/or translational regulatory regions that function in avian cells, such as promoters which direct expression in particular tissues and egg parts such as yolk. The gene regulatory regions can be obtained from a variety of sources, including chicken anemia or avian leukosis viruses or avian genes such as a chicken ovalbumin gene.

Production of PBD fusion proteins in insect cells can be conducted using *baculovirus* expression vectors harboring a PBD fusion protein transgene. *Baculovirus* expression vectors are available from several commercial sources such as Clonetech. As with the other expression systems described above, the timing, extent of expression and activity of the PBD fusion protein transgene can be regulated by fitting the polypeptide coding sequence with the appropriate transcriptional and translational regulatory regions selected for a particular use. Of particular interest are promoter regions which can be induced under preselected growth conditions. For example, introduction of temperature sensitive and/or metabolite responsive mutations into the transgene coding sequences, its regulatory regions, and/or the genome of cells into which the transgene is introduced can be used for this purpose.

The transformed host cell is grown under appropriate conditions adapted for a desired end result. For host cells grown in culture, the conditions are typically optimized to produce the greatest or most economical yield of PBD fusion proteins. Media conditions which may be optimized include: carbon source, nitrogen source, addition of substrate, final concentration of added substrate, form of substrate added, aerobic or anaerobic growth, growth temperature, inducing agent, induction temperature, growth phase at induction, growth phase at harvest, pH, density, and

maintenance of selection. Microorganisms such as yeast, for example, are preferably grown using selected media of interest, which include yeast peptone broth (YPD) and minimal media (contains amino acids, yeast nitrogen base, and ammonium sulfate, and lacks a component for selection, for example uracil). Desirably, substrates to be added are first dissolved in ethanol. Where necessary, expression of the polypeptide of interest may be induced, for example by including or adding galactose to induce expression from a GAL promoter.

Expression in cells of a host animal can likewise be accomplished in a transient or stable manner. Transient expression can be accomplished via known methods, for example infection or lipofection, and can be repeated in order to maintain desired expression levels of the introduced construct (*see* Ebert, PCT publication WO 94/05782). Stable expression can be accomplished via integration of a construct into the host genome, resulting in a transgenic animal. The construct can be introduced, for example, by microinjection of the construct into the pronuclei of a fertilized egg, or by transfection, retroviral infection or other techniques whereby the construct is introduced into a cell line which may form or be incorporated into an adult animal (U.S. Pat. No. 4,873,191; U.S. Pat. No. 5,530,177; U.S. Pat. No. 5,565,362; U.S. Pat. No. 5,366,894; Wilmut *et al.* (1997) *Nature* 385:810). The recombinant eggs or embryos are transferred to a surrogate mother (U.S. Pat. No. 4,873,191; U.S. Pat. No. 5,530,177; U.S. Pat. No. 5,565,362; U.S. Pat. No. 5,366,894; Wilmut *et al.* (*supra*)).

After birth, transgenic animals are identified, for example, by the presence of an introduced marker gene, such as for coat color, or by PCR or Southern blotting from a blood, milk or tissue sample to detect the introduced construct, or by an immunological or enzymological assay to detect the expressed protein or the products produced therefrom (U.S. Pat. No. 4,873,191; U.S. Pat. No. 5,530,177; U.S. Pat. No. 5,565,362; U.S. Pat. No. 5,366,894; Wilmut *et al.* (*supra*)). The resulting transgenic animals may be entirely transgenic or may be mosaics, having the transgenes in only a subset of their cells. The advent of mammalian cloning, accomplished by fusing a nucleated cell with an enucleated egg, followed by transfer into a surrogate mother, presents the possibility of rapid, large-scale production upon obtaining a "founder" animal or cell comprising the introduced construct; prior to this, it was necessary for the transgene to be present in the germ line of the animal for propagation (Wilmut *et al.* (*supra*)).

Expression in a host animal presents certain efficiencies, particularly where the host is a domesticated animal. For production of PBD fusion proteins in a fluid readily obtainable from the host animal, such as milk, the transgene can be expressed in mammary cells from a female host. The transgene can be adapted for expression so

that it is retained in the mammary cells, or secreted into milk, to form the PBD fusion proteins localized to the milk (PCT publication WO 95/24488). Expression can be targeted for expression in mammary tissue using specific regulatory sequences, such as those of bovine α-lactalbumin, α-casein, β-casein, χ-casein, κ-casein, β-lactoglobulin, or whey acidic protein, and may optionally include one or more introns and/or secretory signal sequences (U.S. Pat. No. 5,530,177; Rosen, U.S. Pat. No. 5,565,362; Clark *et al.*, U.S. Pat. No. 5,366,894; Garner *et al.*, PCT publication WO 95/23868). If purification is necessary, the PBD fusion proteins are readily purified by affinity chromatography using a substrate polysaccharide.

In using the subject invention, polysaccharide structures are modified using PBD fusion proteins by contacting a polysaccharide structure with a sufficient amount of the PBD fusion protein for a time sufficient to achieve a desired modification under appropriate conditions of reagents, temperature and the like. Conditions of modification generally are optimized to provide for Km, Vmax, and kcat and other biochemical parameters such as pH optima of the PBD. The interaction of the PBD with substrate generally is extremely rapid. To achieve a desired effect, it therefore is necessary to evaluate various concentrations of PBD fusion protein, and/or time and/or temperature of treatment to achieve a desired effect. The conditions used are determined empirically and are based upon the requirements of the PBD fusion protein used and the desired end result. As an example, typical conditions for PBD fusion proteins comprising a PBD derived from an endoglucanase include mM phosphate, pH 7.0, a concentration of PBD generally of about 0.1-10 mg/ml per 25 mg of cellulose fiber such as cotton. The temperature is generally about 20-37 °C, preferably about 25 °C. The time of treatment varies from 5 minutes to up to 12 hours, although longer treatments may be used so long as the polysaccharide structures are not damaged. Generally as appropriate, the mixture is gently agitated to facilitate uniform treatment of the structures. Following treatment of the structures, the structures are dried and then used for preparation of an end product such as paper or textile. Alternatively or additionally, an end product such as paper or textile is treated by PBD, taking into account considerations similar to those listed above.

An assay of the progress of modification or the rate of reaction can be used for the detection of inhibitory end products that might be formed during the modification treatment and for the detection of intermediate or final desirable properties that are produced during treatment. For example, it may be desirable not to fully crosslink the fibers. Rather, it may be preferable to stop the reaction at an intermediate point to obtain polysaccharide structures having desirable properties that are present due to incomplete crosslinking of the structure, for example to obtain a less rigid yarn for weaving. A number of objective tests are known to those of skill in the art for

evaluating the PBD treatment, including Young's modulus, strain at maximum load, energy to break point, and toughness.

The type of modification of the polysaccharide structure that is achieved depends at least in part upon the nature of the protein that is fused to the binding domain. Modification by a PBD is defined as an observable (detectable) change in the structure of the polysaccharide. This includes aggregation of the polysaccharide structure leading to observable modifications such as increased wet strength, change in surface properties such as hydrophobicity, hydrophilicity, wetability, surface texture and the like. Electrical properties of a polysaccharide containing material that can be changed include surface charge (positive or negative) and electrical conductivity. Chemical properties of a polysaccharide containing material that can be changed include the introduction of various chemically and photochemically reactive chemical groups to at least the surface of the polysaccharide containing material. Mechanical properties of a polysaccharide containing material that can be changed include tensile strength, resistance to shear, abrasion resistance, frictional coefficient, and elasticity. As an example, when the polysaccharide structure is a cellulose, a reagent or composition having two or more CBDs per molecule can be used to cross-link cellulose fibers. Figs. 9A-C schematically represent some of the ways in which a CBD coupler unit of the invention can interact with and bind to a polymeric structural unit of a polysaccharide. Fig. 9A schematically represents a CBD coupler unit having a first CBD bound to a first polymeric structural unit, and a second CBD bound to a second polymeric structural unit. It is to be expected that, at least in the case of a linker unit having a high degree of flexibility is used, both a first and a second CBD of a CBD coupler unit can bind to the same polymeric structural unit. Fig. 9B shows a CBD coupler unit having a flexible linker unit, wherein both the first and second CBDs are bound to a single polymeric structural unit. Fig. 9C schematically represents how a plurality of polymeric structural units can be cross-linked by a plurality of CBD coupler units to form a three dimensional network of polymeric material. In this manner, aggregates of filamentous polysaccharide, for example, cellulose filaments, can be formed. Materials constructed from cellulosic materials, such as paper, cotton yarn and cotton fabric (both woven and non-woven), which are cross-linked via CBD coupler units have altered mechanical properties, such as Young's modulus. Cross-linking of cellulosic materials can be performed at various stages in manufacture of a cellulose-containing material. For example, in the case of paper products, cross-linking can be performed by treating cellulose fibers with a CBD coupler unit composition at various stages in the paper making process, or a formed paper product can be treated with a CBD coupler unit. Similarly, cotton yarn or cotton fabric can be

cross-linked with a CBD coupler unit composition to provide yarn or cotton fabric having improved surface and/or mechanical properties.

By treating a polysaccharide structure or a polysaccharide containing material with a PBD fusion protein comprising a functional moiety, novel materials with a variety of novel physical, electrical, chemical, and mechanical properties can be obtained. Fig. 8 schematically represents a CBD functionating moiety that includes at least one CBD and a functional moiety (FM) attached thereto. The functional moiety can be any of numerous chemical species, including: a hydrophobic moiety, such as a hydrophobic amino acid sequence or peptide or a fatty acid derivative; to decrease wet-ability and to provide increased tolerance of the material to moisture and water, a hydrophilic moiety; an electrically charged or ionic moiety; a silicon binding moiety; a polymer binding moiety; a metal or metal binding moiety to provide for binding to a metal substrate (examples of metal binding proteins include bacterial siderophores, metallothioneins and metallothionein-like proteins (Slice *et al.* (1990) *J. Biol. Chem.* 265: 256-263), ferritin (Spanner *et al.* (1995) *Bone* 17: 161-165), and designed metal-binding proteins (for example, Pessi *et al.* (1993) *Nature* 362: 367-369)); a chemically reactive group; a photo-chemically reactive group; or a thiol group. A chemically reactive group of the invention can include, for example, an aldehyde, a maleimide, a hydrazide, an epoxide, or a carbodiimide. A photo-chemically reactive group of the invention can include a phenylazide.

Similarly, a composition having a hydrophobic moiety, for example, a hydrophobic polypeptide, a long chain hydrocarbon or hydrocarbon derivative, can be used to confer hydrophobicity to cellulose fibers or products made from cellulose fibers. Hydrophobicity leads to decreased wet-ability of a material constructed from the modified cellulose fibers, and indirectly results in increased durability of the material in the presence of water. On the other hand, cross-linking of cellulose fibers directly leads to increased wet strength of the material. For the sake of simplicity, herein reference to strength of paper or other cellulose containing materials is used nonspecifically to include wet strength of such materials.

The types of polysaccharide materials that can be modified using the subject process are varied. Examples include wood products, paper products derived from cellulose fibers, and products derived from cotton or ramie, such as yarn and fabric. The term "paper" includes sheet-like masses and molded products made from fibrous cellulosic materials and combinations of cellulosic materials and synthetic materials. Examples of paper include tissue paper, office paper, newsprint, fluting paper, paper towel, laminated paper, and paperboard. The term "polysaccharide material" or "polysaccharide-containing material" refers to a material that comprises at least one

polysaccharide, generally a substantial amount of least one polysaccharide, such as cellulose, or chitin.

CBD-containing compositions can also find applications in paper making processes. According to the invention, a CBD-containing composition can be used to treat cellulosic material at different stages of a paper making process. For example, treatment may be performed at the forming stage, or at the sizing stage. Treatment at the forming stage of paper making may be performed by adding a CBD cross-linking composition (for example, a CBD coupler unit composition or a cellulose cross-linking (fusion) protein (CCP)) to a suspension of cellulose fibers. Preferably, treatment of cellulosic material with a CBD-containing composition occurs at, or before, the forming stage. A functional moiety can be attached, conjugated, or coupled to a CBD according to methods described hereinabove for attachment of a linker unit to a CBD to form a CBD coupler unit (Figs. 4A-G, 5A-B) (see also, for example, U.S. PAT. NO. 5,962,289).

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by

Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorpotaed by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

Deposit of Biological Materials

E. coli pET-CBD was deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, M, VA 20110-2209 on Apr. 12 1993, and has been assigned the accession number 75444.

Example 1

Construction and Expression of CBDs and CBD Fusion Proteins

The contents of the following U.S. patents, which disclose the construction and expression of various CBDs and CBD fusion proteins, are incorporated by reference herein: U.S. PAT. NO. 5,496,934; U.S. PAT. NO. 5,670,623., U.S. PAT. NO. 5,719,044. ; U.S. PAT. NO. 5,738,984; U.S. PAT. NO. 5,837,814; and U.S. PAT. NO. 5,856,201 all to Shoseyov *et al.*; U.S. PAT. NO. 5,137,819; U.S. PAT. NO. 5,202,247; U.S. PAT. NO. 5,340,731; U.S. PAT. NO. 5,928,917; and U.S. PAT. NO. 5,962,289 all to Kilburn *et al.*; and U.S. PAT. NO. 5,821,358 to Gilkes *et al.*

1.1 Construction and expression of the cellulose binding domain of C. cellulovorans (CBDclos):

Construction and over-expression of the cellulose binding domain of Cellulose Binding Protein A of *C. cellulovorans* in *E. coli* BL12 (DE3) harboring the pET-CBD plasmid (see Figs. 1A-C) has been described by M.A.Goldstein *et al.*, (1993) (*J. Bacteriol.* 175: 5762-5768). Also *see* U.S. PAT. NO. 5,496,934 and U.S. PAT. NO. 5,719,044, both of which are incorporated herein by reference in their entirety.

1.2 Construction and expression of CCP-180:

pET-CCP-180 (Figs. 2A-E) was constructed from pET-CBD (Figs. 1A-C, M.A. Goldstein *et al.* (1993) *J. Bacteriol.* 175: 5762-5768) and pET-CBD-180 (Figs. 1D-G, E. Shpigel *et al.* (1999) *Biotech. Bioeng. 65: 17-23* [pET-CBD and pET-CBD-180 were digested with *Nco*I and *Bam*HI and the resulting DNA fragments separated on 1.2 % and 0.6 % agarose gels, respectively. The 500 bp fragment of pET-CBD and the 5 Kb fragment of pET-CBD-180 were extracted from the gel using a Qiaex DNA gel extraction kit (Qiagen, Inc, California), and the two fragments were ligated. The ligation mixture was transformed into *E.coli* XL1-blue competent cells, followed by transformation into the expression host *E.coli* BL21 (DE3). The positive clone containing two CBDs fused in frame was designated pET-CCP-180 and confirmed by sequencing. Expression of CCP-180 was conducted as described by M.A. Goldstein *et al.* (1993) *J. Bacteriol.* 175: 5762-5768 for CBDclos

1.3 Cloning and Expression of Protein A-CBD:

CBD was PCR amplified using the *cbpA* gene (Shoseyov *et al.*, (1992) *Proc. Natl. Acad. Sci. USA* 89: 3483-3487) as a template: primer A (N-terminal primer): 5'-GGGGGAATTCCATGGCAGCGACAT-3' (SEQ ID NO:11) containing an *Eco*RI site, and primer B (C-terminal primer): 5'-GGGGGATCCTATGGTGCT-3' (SEQ ID NO:12) containing a stop codon followed by a *Bam*HI site. The primers were designed to enable *Eco*RI/*Bam*HI force cloning of the 500 bp DNA fragment of into the plasmid pRIT2, fused in frame to the C-terminal of the Protein A gene. PCR conditions were as described in Innis *et al.*, *PCR Protocols: A Guide to Methods & Applications*. Innis *et al.* Ed., Academic Press, San Diego, 1990) with the following modifications: 2 ng of template DNA and 1mM $MgCl_2$ were used in the reaction mixture. The reaction was conducted using a programmable thermal controller (M&J Research, Inc.,). Standard DNA manipulations were conducted according to Sambrook *et al.*, Eds. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press.

The PCR amplified product was digested with *Eco*RI and *Bam*HI, and the expected 500 bp DNA fragment was isolated from 1.5 % agarose gel using a Qiaex gel extraction kit (Qiagen, Inc.). The *Eco*RI/*Bam*HI fragment was ligated into *Eco*RI/*Bam*HI-predigested pRIT2 using T4 ligase. The ligation mixture was used to transform *E. coli* strain 2097 competent cells, and transformed colonies were selected

on LB agar plates containing 100 mg/L ampicillin. The successful construct containing the DNA insert of interest was designated pRIT2-CBD.

Prot A-CBD was cloned into the T7 mediated over-expression vector pET3d (F. Studier *et al.*, (1986) *J. Mol. Biol.* 189: 113-130). The Prot A-CBD was PCR amplified using pRIT2-CBD as a template using the following primers: C-terminal: as described above (i.e., 5'-GGGGGGATCCTATGGTGCT-3' SEQ ID NO:12); and N-terminal: 5'-GGGGGGTACCATGGAACAACGC-3' (SEQ ID NO:13), containing an initiation site within the *Nco*I site. The PCR product was partially digested with *Nco*I. The recovered DNA was digested with *Bam*HI, and the 1.3 Kb DNA fragment was cloned into pET3d. The ligation mixture was used to transform *E. coli* XL1-Blue competent cells, and transformed colonies were selected on LB agar plates containing 100 mg/L ampicillin. The successful construct containing the DNA insert was designated pET-ProtA-CBD (Figs. 3A-G). pET-ProtA-CBD was transformed into *E. coli* BL21 (DE3) competent cells. Expression of the fusion protein was conducted as described by Nilsson *et al*, (1985), *EMBO J.* 4: 1075-1080. All the cells were grown in shake flasks at 250 rpm in a volume of 40 ml of LB, supplemented with 50 mg/L ampicillin, inoculated with 400 µl of an overnight culture of *E. coli* 2097 containing pRIT2-CBD. The culture was grown at a temperature of 30 °C until it attained an $O.D._{600\ nm}$ of 0.4. The temperature was then raised to 42 °C for 45 minutes, and then decreased to 37 °C for an additional 2 hours.

Over-expression of ProtA-CBD was obtained in *E. coli* BL21 (DE3) harboring pET-ProtA-CBD. Inoculum was prepared by growing the cells overnight in M9 minimal medium (0.65 Na_2HPO_4, 0.3 % KH_2PO_4, 0.255 NaCl, 0.5 % NH_4Cl, 20 % glucose, 2mM $MgSO_4$, 0.1 mM $CaCl_2$ and 1 mM thiamine-HCl) containing 50 µg/ml ampicillin. After diluting the inoculum 1:50 in TB medium (1.2 % bacto-tryptone, 2.4 % bacto-yeast extract, 0.4 % (v/v) glycerol, 0.17 M KH_2PO_4, and 0.72 M K_2HPO_4) containing 100 µg/ml ampicillin, cells were grown at 37 °C to an $O.D._{600\ nm}$ of 1.5, after which 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added. The cells were grown for an additional 4 hours at 37 °C. The cells were harvested by centrifugation at 2,000 g for 10 minutes.

1.4 Purification of ProtA-CBD:

Cells were suspended at a concentration of 0.1 g/ml in 50 mM Tris/HCl, 10 mM EDTA, pH 8, and were disrupted by a RANNIE high pressure laboratory homogenizer (MINI/LAB Type 8.30 H). The suspension was centrifuged, and 1 liter of supernatant at a protein concentration of 5 mg/ml was applied to a cellulose (Avicel 200 Sigma) column (2.6 x 32 cm). The column was equilibrated with PBS (15 mM phosphate buffer, 150 mM NaCl, 3 mM KCl, pH 7.4). The column was washed at a flow rate of 5 ml/min until the absorbancy at 280 nm was less than 0.05. ProtA-CBD

was eluted with 50 mM Tris/NaOH solution, pH 12.5. The eluted ProtA-CBD was immediately titrated to pH 8 with HCl and lyophilized. Total *E. coli* protein (before application to the cellulose column) and the peak (ProtA-CBD) eluted from the cellulose column were analyzed on 12.5 % SDS-PAGE according to Laemmli (U.K. Laemmli (1970) *Nature* 227:680-685). The ProtA-CBD peak showed a single band at about 45 kD.

Example 2

Measurement of Mechanical Properties of Treated (CBD-Modified) and Untreated Materials

Mechanical properties were measured using a universal testing machine (Fig. 11, Instron, High Wycombe, UK) Interface type: 1011 series. Sample rate: 10 pts/sec. Crosshead speed: 5 mm/min. All measurements were taken at 23 °C and 65 % relative humidity.

2.1 Young's Modulus:

Tensile elastic modulus, or Young's modulus, is an important property of materials. Young's modulus may be loosely defined as the force required to elongate a material in the elastic regime using relatively small forces that do not irreversibly stretch the material.

2.2 Paper Treatments:

Rectangular strips of tissue paper (dimensions: 45 mm x 10 mm x 0.1 mm) were treated by immersion for 10 minutes in solutions of CBDclos ,CCP, Prot-A-CBD, Ab-ProtA-CBD- at a concentration of 2.5 mg/ml and 2.0 mg/ml, respectively, in 20 mM Tris base, pH 7. Control treatment consisted of immersion in a solution of 20 mM Tris base, pH 7, also for 10 minutes. After immersion the treated and control strips were removed from the liquids and dried for 2 days under vacuum.

2.3 Results of Paper Treatment:

2.3.1 Young's modulus:

Young's modulus values for control, CBDclos-treated and CCP-180-treated samples of paper are given in Fig. 10A. The paper treated with CBD had a Young's modulus significantly greater than that of the control (untreated) paper. The paper treated with CCP had a Young's modulus even greater than that of the CBD treated paper. These results indicthat treatment of paper with a CBD or with a CCP alter at least one mechanical property of the paper. More specifically, treatment of paper with either CBD or CCP resulted in increased tensile strength (as determined by Young's modulus values) of the treated paper as compared with the untreated paper.

2.3.2 Strain at Maximum Load:

Results showing strain at maximum load for CBDclos treatment and CCP-180 treatment of paper samples are shown in Fig. 10B. Neither CBD treatment nor CCP treatment resulted in substantial change in strain at maximum load, as compared with the control value. These results indicate that treatment of the paper with CBD or CCP did not significantly affect its elasticity.

2.3.3 Energy to Brake Point:

Results showing energy to brake point for paper samples treated with CBD or CCP-180 are shown in Fig. 10C. Energy to breake point of the CBD treated paper was substantially the same as that of the control. However, the paper treated with the crosslinking protein, CCP-180 showed significantly increased energy to brake point as compared with the control.

2.3.4 Toughness:

Results showing toughness for paper samples treated with CBD and CCP are shown in Fig. 10D. Toughness of the CBD treated paper was substantially the same as that of the control. However, the paper treated with CCP again showed significantly increased toughness as compared to the control.

2.4 Yarn Treatments:

The cotton yarn used in this study was 100 % gray cotton double yarn fiber (34/2) with low T.P.U. (turns per inch). Yarn diameter was 0.5 mm and the weight per length was 0.8 mg/cm. In each treatment, the yarn samples were immersed in protein solutions employing a purpose-built yarn treatment apparatus (YTA) of the type known in the art. The yarn treatment apparatus is schematically represented in Fig. 11. The apparatus includes a feeder wheel, a collecting wheel, a first bath A, a second bath B, and an engine engaged with the collecting wheel. The feeder wheel and collecting wheel may be interchanged, thereby allowing yarn to be re-passed through baths A and B. The engine can be operated at various selected speeds, thereby allowing the immersion time of a yarn sample to be determined. Lengths of yarn may be connected between the feeder wheel and the collecting wheel, and the yarn may be moved from the feeder wheel to the collecting wheel by passing the yarn through liquid(s) contained within bath A and bath B. In this way, yarn may be immersed in a single liquid (present in both baths) or in two different liquids for a particular time period.

Lengths of cotton yarn (3-4 meters) were wound onto the feeder wheel of the YTA, one end of the yarn was connected to the collecting wheel, and the yarn was advanced through bath A and bath B by means of the engine. Dipping duration of the yarn was approximately 45 seconds.

Treatments of the cotton yarn described above were as follows:

(i) Treatment with CCP-180: yarn fibers were immersed (bath A) in a solution of CCP-180 (1mg/ml in 20mM Tris base, pH 8).

(ii) Treatment with Protein A-CBD (CBD-PA yarn fibers were immersed (bath A) in CBD-PA solution (0.75 mg/ml in 20mM Tris base, pH 8) and then washed (bath B) in 1XTBS (45 seconds dipping time).

(iii) Dual treatment with Protein A CBD and antibodies: yarn fibers were immersed (bath A) in CBD-PA solution (0.75 mg/ml in 20 mM Tris base, pH 8) and washed (bath B) in 1X TBS (45 seconds immersion time). The collecting wheel was then switched with the feeder wheel, and the yarn was immersed (bath A) in antiserum solution (0.75 mg IgG/ml) and washed (bath B) in 1X TBS.

(iv) Control: yarn fibers were immersed (bath A) in 20 mM Tris base, pH 8 for 45 seconds.

After treatment, all three treated samples and the control were dried for several hours at room temperature.

2.5 Results of Yarn Treatment:

2.5.1 Young's modulus:

Young's modulus values for control, CCP-180-treated, ProteinA-CBD-treated and Ab-ProteinA-CBD-treated samples of yarn are given in Fig. 12A. The yarn treated with CCP-180 and Ab-ProteinA-CBD had Young's modulus values significantly greater than that of the control (untreated) yarn. These data indicate that treatment of yarn with Ab-ProteinA-CBD and CCP resulted in increased tensile strength (as determined by Young's modulus values) of the treated yarn, as compared with the control. Interestingly, the yarn treated with CBD-PA had a Young's modulus value much lower than that of the control yarn. While not intending to be limited by theory, a possible explanation for the decreased Young's modulus for CBD-PA-treated yarn is loosening of cellulose fibers by CBD-PA (see, for example, U.S. PAT. NO. 5,821,358 the contents of which are incorporated by reference herein).

2.5.2 Strain at Maximum Load:

Results showing strain at maximum load for yarn samples treated with CCP-180, CBD-PA, and CBD-PA-Ab are shown in Fig. 12B. Yarn treated with either CCP-180 or CBD-PA-Ab had lower values of strain at maximum load as compared with the control, thus indicating that these treatments rendered the yarn less elastic as compared with the control. The yarn treated with CBD-PA had a strain at maximum load similar to that of the control.

Example 3

Functionalization of materials

3.1 Functionalization of a filter medium for removal of heavy metal species from a liquid:

A CBD functional moiety (see, for example, Figs. 9A-C) is prepared by coupling a CBD to a functional moiety having affinity for a heavy metal, such as a metal-binding protein. A substrate comprising cellulosic material, such as cotton fibers, is treated with the CBD functional moiety under conditions (pH, temperature, ionic concentration, etc.) such that the CBD component of the CBD functional moiety binds to the substrate, whereby the substrate is functionalized by the metal-binding functional moiety to provide a metal binding substrate or filter medium. A stream of liquid containing an excessive level of a heavy metal is passed over the metal binding filter medium, whereby the concentration of the heavy metal in the liquid stream is greatly decreased to a non-toxic level.

3.2 Functionalization of cellulose fibers for making packaging paper product with decreased wet-ability:

A CBD functional moiety is prepared by coupling a CBD to a hydrophobic functional moiety. Cellulose fibers suitable for paper making are treated with the CBD hydrophobic functional moiety under conditions (pH, temperature, ionic concentration) such that the CBD component of the CBD functional moiety binds to the cellulose fibers to provide cellulose fibers having a hydrophobic moiety attached thereto. Paper produced from the treated cellulose fibers is hydrophobic and resistant to water.

In an alternative example, paper produced from untreated (non-functionalized) cellulose fibers is functionalized with a CBD-linked hydrophobic moiety, either before or after drying the paper. Paper treated with the CBD hydrophobic functional moiety is hydrophobic and resistant to water.

3.3 Functionalization of cellulose fibers for making tissue paper having increased wet-ability:

A CBD functional moiety is prepared by coupling a CBD to a hydrophilic functional moiety. Tissue paper is treated (functionalized) with the CBD hydrophilic functional moiety, either before or after the first or second drying stages of a paper making process. Tissue paper treated with the CBD hydrophilic functional moiety is hydrophilic and shows increased absorption of water and aqueous liquids.

Example 4

Expressing S-protein-CBD-S-peptide (SSC)

Fig. 13 shows the results of expression of SCS in *E.coli*.. *E.coli* proteins before induction with IPTG are shown in lane 2, total *E.coli* proteins after induction with

IPTG are shown in lane 3, whereas and inclusion bodies containing the SCS protein are shown in lane 4.

Example 5

Treatment of preformed paper by CBD, CCP or SCS

Fig. 14 shows a Young's modulus map of the results of treating Whatman papers with CBD, CCP, or SCS. Note that treatment of Whatman papers with CBD or CCP in all concentrations tested resulted in increased Young's modulus.

Fig. 15 shows the energy to break points of CBD, CCP, and SCS treated Whatman papers. Note that use of CCP in the concentration of 2.5 mg/ml resulted in about 30 % increase in the energy to break point. In addition, treatment with SCS at all concentrations tested resulted in increased energy to break point.

Fig. 16 shows the results of a toughness test of CBD, CCP, and SCS treated Whatman papers. Note that use of CCP in the concentration of 2.5 mg/ml resulted in about 40 % increase in toughness. In addition, treatment with SCS at all concentrations tested resulted in increased toughness.

Fig. 17 shows the stress at maximum load of CBD, CCP, and SCS treated Whatman papers. Note that all the treatments tested resulted in increased stress at maximum load. The most significant effect was obtained with CCP in the concentration of 2.5 mg/ml. The increase in the stress at maximum load demonstrates an increase of paper strength.

In another set of experiments the effects of CBD and CCP on pre-formed Whatman papers were determined.

Rectangular pieces of Whatman paper No. 1, 40 × 10 mm and 0.18 mm thick (Whatman, Maidstone, England) were immersed for 10 min in a solution (20 mM Tris base, pH 7) containing 2.5 mg/ml CBD or CCP. The samples were then dried for 24 hours in 65 % relative humidity at 23 °C. The final water content in the papers was 3.2 %. Mechanical properties were evaluated according to the international standard testing method for paper and board tensile properties (ISO 1924-2). Tensile testing of the treated papers was carried out using an Instron Universal Testing Machine (UTM) Model 1011 (High Wycombe, UK) in tensile mode. The rectangular papers were inserted into the upper and lower tensile grips (screw-action grips, Instron Corp., Canton, MA) to enable a proper grip during the tension experiments. All

measurements were taken at 23 °C, 65 % relative humidity and a constant deformation rate of 20 mm/min. The tensile properties measured included stress at failure, strain at failure, stretch at break point and energy absorption. All calculations were performed according to Hayden, W., Moffatt, W.G. & Wulff, J. *Mechanical Behavior*. 1–22 (Johan Wiley & Sons, Inc., NY; 1956) and Dufresne, A., Cavaille, J.Y. & Vignon, M.R. Mechanical behavior of sheets prepared from sugar beet cellulose microfibrils. *J. Appl. Polym. Sci.* 64, 1185–1194 (1996).

The stress (σ) was calculated according to equation 1:

$$\sigma = F/S \quad (1)$$

where F is the applied load and S is the cross section. S is determined by assuming that the total volume of the sample remains constant, such that:

$$S = S_0 \times l_0/l \quad (2)$$

where S_0 is the cross section at zero time. The strain (ε) can be determined by:

$$\varepsilon = \ln(l/l_0) \quad (3)$$

where l and l_0 are the length during the test and the length at zero time, respectively. The data allow the plotting of stress versus strain curves, and the calculation of Young's modulus (E):

$$E=\Delta\sigma/\Delta\varepsilon \quad (4)$$

The values reported below are averages of at least 15 measurements.

Fig. 18 shows typical stress versus strain curves of pre-formed Whatman papers treated with CBD or CCP. The deformation behavior of the treated paper under an applied load could be deduced from the stress-strain curve. Up to 0.02 strain, a linear relationship between stress and strain was observed. However, at strains higher than 0.02, a nonlinear relationship was found. It is evident from Fig. 18 that the tensile stress increases from control to CBD and to CCP, respectively. The tensile strength value of the CCP-treated paper was about 40 % higher than the non-treated paper and 14 % higher than the CBD-treated paper. The CBD-treated paper strength was about 25 % higher than that of the non-treated paper. In both treatments, the differences were statistically significant (Table 6).

Table 6

	control	CBD 2.5 mg/ml	CCP 2.5 mg/ml
Stress at failure (MPa)	7.4^{c}	9.2^{b}	10.5^{a}
Strain at failure (%)	10.4^{b}	11.7^{ab}	15.5^{a}
Young's Modulus (MPa)*	183.3^{b}	197.2^{ab}	214.5^{a}
Energy at failure (10^3J/m^3)	0.208^{c}	0.254^{b}	0.418^{a}

* Young's modulus was calculated at 3 % deformation. Values in a row followed by a different letter superscript differ significantly at p = 0.01.

The changes in paper-failure strain are also significant. In paper treated with CCP, the strain to failure was increased by about 50 % relative to the non-treated paper. The effect of CBD was less significant and resulted in only a 12 % increase. Treating paper with CBD or CCP produced a less brittle paper. The Young's modulus of treated paper, derived from the initial slope of the stress-strain curve (linear until 3 % deformation), is summarized in Table 1. Treating papers with CCP resulted in a 17 % increase in their Young's modulus while CBD treatment resulted in only a 7.5 % increase. Energy absorption is determined by calculating the area under the stress-strain curve and the results are summarized in Table 6. The trend observed in the data for tensile strength applied for the energy absorption; however, the magnitude was bigger. The energy absorption of the CCP-treated paper was about 100 % higher than that of the control while that of the CBD treated paper was only 23 % higher. The value for CCP treated paper was about 64 % higher than that for its CBD-treated counterpart. In all tested parameters the effect of CCP was statistically significant, whereas treatment with CBD resulted in statistical significance only for stress at failure and energy absorption (Table 6).

Fig. 19 shows water-absorption time of pre-formed Whatman papers treated with CBD or CCP at different concentrations. Fig. 20 shows time-lapse photographs of water absorption on pre-formed Whatman paper treated with CCP. Distilled water (10 µl) was pipetted onto the treated papers and the time to full absorption was measured in seconds. Water absorption was also visualized using an optical contact angle meter, CAM2000 (KSV Instruments, Helsinki, Finland). One drop of water was dripped onto paper samples and pictures were taken with time lapses of 20 ms. The first frame was taken 25 ms after the water had come into contact with the paper. In

non-treated paper, absorption time was less than a second. Water absorption time of CBD- and CCP-treated papers increased with increasing protein amount. When CCP was applied at a concentration of 2.5 mg/ml, water absorption time was two orders of magnitude higher than with paper treated with CBD at the same concentration (580 seconds for CCP versus 5 seconds for CBD) and at least four orders of magnitude higher then the non-treated paper (580 seconds for CCP versus less than a second for the control).

Optical contact angle meter (CAM) was used to visualize the dynamics of water dropped onto CCP-treated paper. Fig. 20 (photograph F) illustrates the contact of the water droplet with non-treated paper after 25 ms. It is clear that water absorbs into the paper immediately after contact. Fig. 20 (photographs A to E) further illustrates the absorption of a water droplet by CCP-treated paper versus lapsed time. In the first 2 minutes, no absorption could be detected, and the contact angle remained at > 90 °. Only after 4 min was absorption into the paper observed. Even after 8 min, the water was not completely absorbed by the paper. Full absorption into the paper was observed only after 10 min (Fig. 19).

Example 6

Cross-linking of fine cellulose fibers prior to the forming step of a paper making process

A CBD coupler unit composition or reagent is prepared by linking at least two CBDs with a linker unit. A suspension of cellulose fibers, which includes a substantial amount of fine cellulose fibers capable of passing through the forming fabric (filter), are treated with the CBD coupler unit composition prior to passing the suspension through the forming fabric. CBD coupler units of the CBD coupler unit composition crosslink with the fine cellulose fibers to form a plurality of three-dimensional aggregates of cellulose fibers. After passage of the treated suspension through the forming fabric, the three-daggregates of cellulose fibers are retained by the filter, thereby allowing for greatly enhanced recovery of raw material (cellulose fibers).

The above results demonstrate that PBD fusion proteins that include a dual or dimeric PBD (e.g., a fusion product of two CBDs, for example, cellulose cross-linking protein (CCP)), a fusion product of a CBD with Protein A, and a Speptide-CBD-Sprotein fusion can be prepared and used to modify polysaccharide structures.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.